THE ROLE OF APOPTOSIS IN SARCOPENIA: THE EFFECTS OF AGE AND CALORIC RESTRICTION ON SKELETAL MUSCLE APOPTOSIS

By

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THE ROLE OF APOPTOSIS IN SARCOPENIA: THE EFFECTS OF AGE AND CALORIC RESTRICTION ON SKELETAL MUSCLE APOPTOSIS

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Sarcopenia may be partly due to a loss in total fiber number by apoptosis. We have investigated age-related alterations in apoptotic stimuli, such as mitochondrial dysfunction, as well as changes in apoptotic markers and adaptations in apoptotic pathways. We studied apoptosis in the gastrocnemius muscle focusing on the mitochondrial-mediated apoptotic pathway, including caspase-independent apoptosis by apoptosis inducing factor, the endoplasmic-reticulum-mediated pathway, and the receptor-mediated pathway. We report that the rate of apoptosis does increase in aged muscle tissue and that there is a highly significant correlation between the apoptotic index and muscle mass suggesting that apoptosis does contribute to sarcopenia. Many adaptations occur in the mitochondrial-mediated and endoplasmic-reticulum-mediated apoptotic pathways, but not the receptor-mediated pathway. We found that age-related alterations in the mitochondrial-mediated pathway act to

protect against apoptosis and that this pathway may be inhibited. Alterations in the endoplasmic-reticulum-mediated pathway may contribute to apoptosis and loss of fibers with age.

Caloric restriction is a powerful anti-aging intervention. Therefore, we used caloric restriction as an intervention to reduce oxidative stress and mitochondrial dysfunction and to prevent fiber loss with age. We found that lifelong caloric restriction attenuated the rate of apoptosis in aged muscle tissue and generally reversed most of the age-related alterations in the apoptotic pathways studied. However, we did not detect any effects on the receptor-mediated pathway. Our results suggest that apoptosis does contribute to age-related fiber loss and that the endoplasmic-reticulum-mediated apoptotic pathway may be playing a significant role in the pathogenesis of sarcopenia.

CHAPTER 1
INTRODUCTION

Significance

Sarcopenia is a condition associated with aging characterized by the loss of muscle mass and strength. Weakness in the elderly can lead to an increased incidence of falls and injuries and loss of independent living. Furthermore, loss of muscle mass equates to loss of nutritional reserves (protein and glycogen) impairing capacities of the immune response[1]. Loss of muscle mass may also reduce the ability to maintain body temperature by shivering thermogenesis in older individuals[2]. By the year 2030 the elderly population will grow from 13% to ~20% and it is estimated that $130 billion will be imposed by physical frailty. Elucidating mechanisms of sarcopenia and applying interventions can reduce the severity of sarcopenia and eliminate the physical consequences and associated medical costs.

Skeletal muscle mass decreases with age via a decrease in the number of fibers and atrophy of the remaining fibers[3] resulting in muscle weakness. Atrophy of myofibers contributing to the loss of muscle mass has been studied extensively. It has been postulated that increased levels of oxidative stress in aged muscle is a key factor involved in myofiber atrophy, since aged skeletal muscle may have increased production of oxidants and oxidative damage compared with young muscle[4, 5, 6].

The role of cell death and loss of muscle fibers in sarcopenia has been ill investigated. Loss in cell number with age may occur via necrosis and/or apoptosis.

Necrosis is a form of cell death characterized by inflammation and damage to surrounding tissue, while apoptosis is a form of cell suicide triggered by a stimulus that activates a specific set of signaling pathways. Apoptosis is a "quiet" death that does not elicit an inflammatory response and does not disturb neighboring cells. Whether a cell undergoes necrosis or apoptosis depends on the severity of the stimulus or insult. Severe insults usually result in necrosis while mild insults induce apoptosis. For example, a mild oxidative stress or mild decrease in ATP levels leads to apoptosis whereas severe oxidative stress or depletion of ATP levels results in extensive cellular damage and necrosis[7].

Apoptosis plays an important role in development and maintenance of health. For instance, the developing fetus loses the webbing between the toes and fingers via apoptosis. Furthermore, as cells continually divide and proliferate throughout life, such as the epithelial cells of the digestive tract and epidermis of the skin, the old cells perish via apoptosis. Apoptosis can also be a protective or homeostatic mechanism. Specifically, cells infected by a virus are killed via this cell suicide program.

When the apoptotic process is not properly regulated, resulting in excess or deficient apoptosis, disease may occur. Alzheimers, Parkinsons, and other degenerative diseases are characterized by excessive apoptotic cell death, while cancer and some autoimmune disorders are characterized by deficient apoptosis.

Apoptosis may also contribute to the normal aging process. Various organs constituting post-mitotic cells, such as the heart and the brain, lose cells with age, in part, via the apoptotic process[8,9]. Cell loss in these respective organs is thought to

contribute to cardiac dysfunction and dementia associated with advancing age. It is not known, however, if the apoptotic process contributes to the loss of muscle fibers and therefore muscle mass and weakness with age.

Apoptotic stimuli and signaling pathways have been extensively studied and elucidated in various cell types, but efforts have been neglected in skeletal muscle. Relatively speaking, very little is known about the role that apoptosis plays in skeletal muscle development, homeostasis, and disease, especially the stimuli and signaling pathways involved. The following studies will be the first to elucidate possible stimuli and signaling pathways relevant in the aging process of skeletal muscle.

These studies will also investigate a possible intervention that may reverse any age-related alterations in apoptotic stimuli and signaling pathways. Caloric restriction is the only intervention to consistently increase mean and maximum life span in mammals. Animals that are restricted in their caloric food intake by 40%, while maintaining their nutritional requirements, live approximately 30% longer than their *ad libitum* counterparts[10]. Caloric restriction attenuates many of the effects of aging, including oxidative damage to proteins, lipids, and DNA[11-13], mitochondrial dysfunction[5,14], muscle contractile dysfunction[15,16], and loss of muscle fibers[5,17]. Therefore, we are using caloric restriction as a means to slow the aging process in order to give us more insight into the role of apoptosis in sarcopenia.

Questions and Hypotheses

Study 1

Question 1. Is apoptosis involved with the skeletal muscle fiber loss with age?

Hypothesis 1. Aged skeletal muscle will be characterized by an increased rate of apoptosis, which will be determined by a measure of mono- and oligo-nucleosomes.

There is only one study that evaluates the effects of aging on skeletal muscle apoptosis and found an increased number of TUNEL positive nuclei and decreased number of striated muscle cells in the urethral rhabdosphincter [18]. We predict a similar phenomenon in locomoter skeletal muscle.

Question 2. Is the mitochondrial-mediated apoptotic pathway involved with the loss of muscle fibers with age?

Hypothesis 2. The mitochondrial-mediated pathway is activated in aged skeletal muscle. Proteins involved in this pathway will be altered to favor apoptosis.

This will be assessed by measuring cytosolic cytochrome c, mitochondrial Bcl-2 and Bax, and caspase-3 activity.

The Bcl-2 family of proteins regulates the release of apoptogenic proteins, such as cytochrome c, from the mitochondria. Mitochondrial dysfunction may stimulate alterations in the mitochondrial membrane Bcl-2 protein content favoring release of apoptogenic factors. This will in turn activate procaspase-9 followed by procaspase-3.

Study 2

Question 1. Does caloric restriction attenuate age-related mitochondrial dysfunction, a known apoptotic stimulus?

Hypothesis 1. Caloric restriction will reduce mitochondrial dysfunction in aged muscle.

Mitochondrial oxygen consumption, ATP production, and hydrogen peroxide production will be measured.

Mitochondrial function deteriorates with age in skeletal muscle[14,5] and has been shown to be a stimulus for apoptosis in many cell types. Oxidants may play a role in this age-related alteration in mitochondrial function. There is some evidence that mitochondrial H_2O_2 production increases with age[4]. We predict that increased oxidant production and mitochondrial dysfunction with age will be attenuated with caloric restriction, thereby relieving a possible stimulus for apoptosis.

Question 2. Does caloric restriction reduce apoptosis in aged skeletal muscle?

Hypothesis 2. Caloric restriction will reduce the rate of apoptosis in aged skeletal muscle.

Caloric restriction attenuated fiber loss with age in the vastus lateralis muscle[5] but the mechanism is unknown. There are no studies evaluating the effects of caloric restriction on apoptosis in skeletal muscle.

Question 3. What apoptotic pathways does caloric restriction alter in aged skeletal muscle?

Hypothesis 3. Caloric restriction will alter the mitochondrial-mediated, sarcoplasmic-reticulum-mediated, and receptor-mediated signaling pathways as well as the caspase-independent pathway involving apoptosis inducing factor (AIF) in such a way to prevent apoptosis.

Aged skeletal muscle is characterized by mitochondrial dysfuncion, loss of calcium homeostasis and increased resting intracellular calcium levels, and increased

tumor necrosis factor-α (TNF-α)[19]. These alterations are all known stimuli for apoptosis resulting in the activation of the respective caspase-dependent apoptotic pathways as well as the caspase-independent pathway (AIF). No studies have investigated these pathways in skeletal muscle. We also predict a decrease in the content of apoptotic inhibitors with age making skeletal muscle more susceptible to apoptosis

Summary

Sarcopenia is in part due to a loss in skeletal muscle fibers. The role of apoptosis in this age-related muscle loss is unknown and will be investigated along with possible stimuli and signaling pathways. We will also investigate the effects of caloric restriction, an intervention that slows the aging process, to offer more insight as to the role of apoptosis in aging muscle.

CHAPTER 2
REVIEW OF LITERATURE

Sarcopenia is a manifestation of myofiber death and atrophy of the remaining fibers. The cause of fiber loss and atrophy remains to be elucidated, but the mitochondrial free radical theory of aging is a well supported school of thought.

The Mitochondrial Free Radical Theory of Aging

The mitochondrial free radical theory of aging was first proposed by Harman in 1972. It was hypothesized that degenerative diseases and aging stem from an accumulation of oxidative damage to mitochondrial DNA (mtDNA) and proteins in post-mitotic somatic cells, such as cardiac muscle, skeletal muscle, and neurons, disrupting the bioenergetic production of ATP which is vital to the cell[20]. MtDNA and proteins of the electron transport chain (ETC) are very susceptible to oxidative damage since they are in close or exact proximity to the site of superoxide production, which is a normal by-product of aerobic metabolism via the ETC itself.

In young healthy individuals, 3-5 % of oxygen consumed is converted to superoxide. Importantly, aging tissues have an increased production of superoxide and hydrogen peroxide (H_2O_2)[21], as well as insufficient antioxidant defenses to protect against these radicals[22]. An imbalance between radical production and antioxidants can increase protein oxidation and mtDNA damage seen with advancing age[23-26].

Individual cells contain hundreds of mitochondria, and each mitochondrion contain 2-10 copies of mtDNA. Mitochondrial DNA encodes 13 proteins that are essential to the ETC, 22 transfer RNAs and 2 ribosomal RNAs. Deletions or mutations in the genome may alter transcription or translation of the ETC proteins producing a "leaky" ETC[27-29] resulting in excessive superoxide radical production inducing further mtDNA damage. It is proposed that this positive feedback loop (Fig. 1) destroys the bioenergetic capacity of the cell decreasing the production of ATP. Disturbance in energy production is detrimental to the cell and eventually to the function of the tissue or organ[27-29].

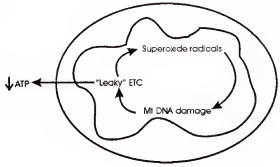

Fig 1. Scheme of mitochondrial free radical damage. Free radical damage to mitochondrial DNA produces a "leaky" electron transport chain resulting in excessive superoxide production and decreased ATP production .

The mitochondrial free radical theory of aging may be applicable to skeletal myofiber atrophy. Skeletal muscle fibers manifest ETC abnormalities and mtDNA deletions with age. Myofiber sections containing ETC abnormalities were atrophied compared to areas of the same fiber without ETC abnormalities[5]. This suggests that

mitochondrial dysfunction may be a contributing factor in the age-related loss of muscle mass.

It is not known whether the mitochondrial dysfunction described to cause atrophy in surrounding areas of the muscle fiber can also trigger apoptosis and death of the fiber.

Apoptosis

Apoptosis is an active process executed via specific signaling events. Apoptosis is characterized by cellular condensation while maintaining intra-organelle integrity, membrane blebbing, DNA fragmentation into oligo-and mono-nucleosomes, destruction of the cytoskeleton, and formation of apoptotic bodies, which macrophages and neighboring cells engulf via endocytosis. Destruction of the cell is mediated by the activation of cysteine proteases, caspases, which cleave each other and other proteins after an aspartate residue within a specified amino acid sequence. There are approximately 14 caspases identified that participate in the apoptotic process depending on the signaling pathway.

Many stimuli inducing apoptosis and signaling pathways have been described in various cell types. The major apoptotic signaling pathways include the following: 1) mitochondrial-mediated, 2) endoplasmic-reticulum-mediated, 3) receptor-mediated, and 4) nuclear-mediated. Various stimuli that trigger apoptosis include the aforementioned oxidative stress and mitochondrial dysfunction, elevated intracellular Ca^{2+}, various cytokines such as TNF-α, and excessive DNA damage.

Apoptotic Signaling Pathways

Mitochondrial Dysfunction and Mitochondrial-mediated Apoptosis

Mitochondrial function decreases with age in various tissues such as brain, heart, and skeletal muscle[5, 30, 31, 32]. Oxidative stress may be intimately involved with mitochondrial dysfunction in aging skeletal muscle. Aged skeletal muscle mitochondria produce more hydrogen peroxide compared with younger counterparts[4]. The chronic oxidant production could cause significant amounts of mitochondrial DNA deletions/mutations affecting energy production. Several groups have shown that various ROS can induce mtDNA mutations that are similar to those found in aging tissue, including skeletal muscle[33, 34, 35, 36]. This may mean that enhanced chronic oxidant production *in vivo* by the mitochondria in aged tissue could be causing the mtDNA deletions/mutations that accumulate with age and therefore elicit mitochondrial dysfunction[37].

Mitochondrial dysfunction can cause tissue deterioration. Isolated mitochondria from fibroblasts of old animals have been microinjected into young cells: this resulted in cellular degeneration[38,39]. These data demonstrate that mitochondria play a central role in cellular maintenance. In addition, Lee et al. reported that the areas of skeletal muscle fibers with ETC abnormalities and mtDNA deletions were atrophied compared with sections of the same fiber without ETC abnormalities[5]. This suggests that mitochondrial dysfunction may be a contributing factor in the age-related loss of muscle mass.

Mitochondria are central to apoptosis. Mitochondrial dysfunction induced by hydrogen peroxide, peroxynitrite, or doxorubicin in vitro has been shown to initiate

the apoptotic process in various cell types such as neurons, endothelial cells, and cardiomyocytes[40-42]. Mitochondrial dysfunction in vivo has also been shown to induce apoptosis. Wallace's group knocked out one allele of the manganese superoxide dismutase (MnSOD) gene rendering these mice to elevated reactive oxygen species and oxidative stress[37]. At sacrifice (~26 months) they found that mitochondrial dysfunction in the liver was elevated in the MnSOD knockout mice compared with control mice of similar age. Furthermore, the rate of apoptosis was also elevated in these mice. The authors also note that the wild-type mice experienced these same alterations, but later in life. These results demonstrate the importance of oxidative stress and mitochondrial dysfunction in the aging process and its role in apoptosis. However, it is not known if mitochondrial dysfunction in aged muscle tissue may result in the activation of the mitochondrial-mediated apoptotic signaling pathway resulting in death of myofibers with age.

Mitochondrial-mediated apoptosis is initiated by the release of cytochrome c from the mitochondria into the cytosol. Cytochrome c then forms an apoptosis initiating complex (apoptosome) with ATP, Apaf-1, and procaspase-9, resulting in activation of this caspase. The active caspase-9 cleaves and activates procaspase-3. This, in turn, activates a cascade of caspases, internally destroying the cell. Some of the targeted proteins of caspase-3 are procaspase-6 and procaspase-7; poly ADP ribose polymerase (PARP); inhibitors of DNA fragmentation factor (DFF) and caspase activated DNase (CAD) resulting in DNA fragmentation; and cytoskeletal proteins such as lamin, fodrin, and actin resulting in cellular condensation and membrane blebbing.

The release of cytochrome c is regulated by the Bcl-2 family of proteins. These include Bax, Bad, Bak, and Bid, which are pro-apoptotic proteins that favor cytochrome c release, and Bcl-2 and Bcl-X_L, which are anti-apoptotic proteins that inhibit cytochrome c release.

How the Bcl-2 family proteins regulate cytochrome c release is currently under investigation. Three hypotheses exist explaining how cytochrome c may be released from the mitochondria: 1.) Physical rupture of the outer mitochondrial membrane, 2.) A channel formed by pro-apoptogenic Bcl-2 family members such as Bax, and 3.) Opening of a pore via a membrane permeability transition (MPT) characterized by loss of the mitochondrial membrane potential[43,44]. Most data support the latter two hypotheses[43,45]. Cytochrome c release has been observed with and without MPT, so which occurs *in vivo* is unknown. Whether or not MPT occurs, Bcl-2 and Bcl-X_L inhibit the release of cytochrome c *in vitro* while Bax induces cytochrome c release[45,46].

The Bcl-2 family member, Bad, acts as a pro-apoptotic protein. The dephosphorylated form of Bad associates with Bcl-2 and Bcl-X_L, inhibiting their anti-apoptotic function. Growth factors promote cell survival via phosphorylation of Bad, resulting in the dissociation from Bcl-2 and Bcl-X_L and the association with a cytosolic adapter protein 14-3-3τ[47]. Bcl-2 and Bcl-X_L can proceed to carry out their anti-apoptotic function once Bad dissociates.

Mitochondria also release apoptosis inducing factor (AIF), which is a caspase-independent mitochondrial death effector. AIF can only be released upon MPT[46]. This pro-apoptotic factor translocates to the nucleus where it induces chromatin

condensation and large scale DNA fragmentation into approximately 50 kbp[48]. Kinetic studies revealed that AIF is released before cytochrome c and its release can also be inhibited by Bcl-2[48]. See Fig 2 for an illustration of the mitochondrial-mediated apoptotic signaling pathway.

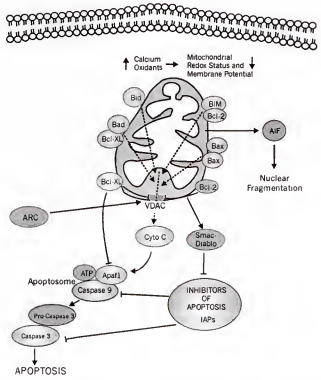

Fig 2. Mitochondrial-mediated pathway of apoptosis. Mitochondrial dysfunction could be caused by factors, such as oxidants, increases in Ca^{2+} levels, a decline in redox status (i.e., glutathione, ATP, NADH), and collapse of mitochondrial membrane potential. Mitochondrial proteins, such as Bcl-2, Bcl-X_L, Bid, and Bax, and their specific ratios ("check point proteins" for cell death) can influence the mitochondrial outer membrane channel, the voltage-dependent anion channel (VDAC), which leads to the release of cytochrome c from the mitochondria. Cytochrome c release could lead to the formation of the "apoptosome" (Apaf-1, caspase-9, dATP), resulting in apoptosis. Other proteins released from the mitochondria, such as apoptosis-inducing factor (AIF), are caspase-independent and translocate to the nucleus, causing large-scale DNA fragmentation. Smac-DIABLO can be released simultaneously with cytochrome c and functions to repress inhibitors of apoptosis proteins (IAPs). Signals and proteins responsible for apoptosis may vary remarkably among cell types. Figure taken from Pollock et al.[44]

Intracellular Calcium and Endoplasmic-reticulum-mediated Apoptosis

The endoplasmic reticulum (ER), called the sarcoplasmic reticulum (SR) in muscle tissue, functions in protein synthesis and packaging as well as a storage site for calcium. Resting cytosolic Ca^{2+} levels are maintained at the micromolar level with a Ca^{2+} gradient of 10,000x across the plasma membrane and endoplasmic reticulum, which is essential for intracellular signaling such as muscle contraction, neurotransmission, neuronal plasticity, and reactions involved in energy and biosynthetic metabolism[19]. Low levels of intracellular Ca^{2+} are maintained by Ca^{2+} pumps and transporters in the plasma membrane, ER membrane, and mitochondrial membrane. The plasma membrane and the mitochondrial membrane contain Ca^{2+}/Na^{+} antiporters and Ca^{2+} symporters. The plasma membrane also contains Ca^{2+}-ATPases as does the ER. Calcium release from the SR during stimulation of muscle contraction occurs via ryanodine receptors (RyR) located in the terminal cisternae while the ER Ca^{2+}-ATPase pumps Ca^{2+} back into the terminal cisternae. Calmodulin (CaM) is a cytosolic calcium sensor that regulates several enzymes, receptors, and channels associated with signaling and metabolism. For example, CaM enhances activity of Ca^{2+}-ATPases and inhibits activity of RyR when Ca^{2+} levels are high, functioning as a feedback inhibitor of Ca^{2+} release[19]. CaM can also stimulate Ca^{2+} release when Ca^{2+} levels are low by activating RyR. Therefore, CaM is a central player in the regulation of cytosolic Ca^{2+} [19].

Calcium homeostasis decreases with age and, therefore, may explain the increase in resting intracellular Ca^{2+} in senescent animals[19, 49]. With so many proteins and cellular compartments involved in calcium homeostasis the mechanisms

contributing to the loss in calcium homeostasis have been difficult to elucidate *in vivo*, but several possibilities exist. First, changes in the reduction/oxidation (redox) status of aged muscle may contribute to the loss in Ca^{2+} homeostasis. Oxidation of essential cysteine residues on CaM has been reported in aged Fischer 344 rats as well as nitration of essential tyrosine residues on the SR Ca^{2+}-ATPase[50,51]. These post-translational modifications can alter Ca^{2+} homeostasis *in vitro*. Secondly, mitochondrial dysfunction (depressed ATP production) may result in lower activity of Ca^{2+}-ATPases making it difficult to maintain low levels of cytosolic Ca^{2+}. Thirdly, mitochondrial dysfunction (decreased mitochondrial transmembrane potential) can alter the ability of the mitochondria to act as a sink for Ca^{2+}. All of these age-related alterations may contribute to the loss in calcium homeostasis and result in elevated intracellular calcium levels.

Intracellular calcium can activate calcium-sensitive endonucleases such as DNase I, proteases such as calpains, and calcium-dependent nitric oxide synthases (NOS) such as neuronal NOS and endothelial NOS increasing the production of nitric oxide. Nitric oxide can directly inhibit ATP production by reversibly inhibiting enzymes of the ETC, such as cytochrome c oxidase. Furthermore, nitric oxide can react with superoxide to form peroxynitrite ($ONOO^-$), a potent reactive nitrogen species (RNS) that can cause oxidative damage to proteins lipids, and DNA. For example, peroxynitrite can irreversibly damage proteins of the electron transport chain and inhibit ATP production causing mitochondrial dysfunction and cell death.

Increased cytosolic Ca^{2+} and ER stress can stimulate apoptosis via the activation of procaspase-12, however we do not know if this is the case in skeletal

muscle. No investigations have been conducted to determine if calcium dyshomeostasis may contribute to skeletal muscle apoptosis. Although very little is known about the caspase-12 associated apoptotic pathway(s), two mechanisms have been described. The first pathway described is mediated by increases in intracellular calcium and the activation of m-calpain, a Ca^{2+} activated neutral protease[52]. The activated m-calpain translocates to the cytosolic side of the ER membrane where procaspase-12 resides, cleaving the caspase to its active form[52]. The second pathway described is mediated by the activation of caspase-7 in response to ER stress induced by thapsigargin, an inhibitor of ER Ca^{2+}-ATPase, which in turn activates caspase-12[53].

Bcl-2 may regulate ER-mediated apoptosis, aside from the mitochondrial-mediated apoptosis. Bcl-2 regulates Ca^{2+} efflux in an unknown manner to prevent apoptosis induced by thapsigargin[54]. Overexpression of Bcl-2 slows Ca^{2+} leakage from the ER and prevents apoptosis, but the mechanism is still to be elucidated[54].

Another regulatory mechanism of apoptosis by the ER may be the controlled release of DNase, involved in nuclear DNA degradation. During apoptosis the DNase gains access to the nucleus, which may be ER-mediated, although signaling pathways are not yet known[54]. See Fig 3 for an illustration of ER-mediated apoptosis.

TNF-α and Receptor-mediated Apoptosis

TNF-α is a pleiotropic cytokine that is produced by many cell types including activated macrophages, lymphocytes, and skeletal myofibers. TNF-α plays a central role in immune and inflammatory responses. TNF-α signals via two membrane receptors called tumor necrosis factor receptor 1 and 2 (TNF-R1 and TNF-R2,

respectively). These receptors are homologous in their extracellular domains, but are structurally different in their cytoplasmic domains[55]. TNF-R1 contains a death domain, whereas TNF-R2 does not[55]. TNF-R1 mediates signaling for both apoptosis and cell survival. TNF-α binding to TNF-R1 can induce apoptosis in an effector cell by the activation of procaspase-8, which cleaves and activates procaspase-3 and initiates the caspase cascade[56]. Alternatively, binding of TNF-α to TNF-R1 can induce a pro-inflammatory/anti-apoptotic response mediated through the cytosolic transcription factor NF-κB. The presence and/or recruitment of adaptor proteins to TNF-R1 determine the outcome: caspase activation resulting in apoptosis or NF-κB activation resulting in cell survival. Adaptor proteins include TNF receptor apoptosis death domain (TRADD), Fas induced apoptosis death domain (FADD), and TNF-R2 associated factor 2 (TRAF2). To activate NF-κB promoting cell survival, TRADD associates with TNFR1 and is able to recruit TRAF-2 from the TNFR2 receptor to form a TNFR1-TRADD-TRAF2 complex[57]. NF-κB promotes cell survival by inducing expression of anti-apoptotic proteins including inhibitor of apoptosis proteins (IAPs), cFLIP, and TRAF2[58]. To induce apoptosis, TRADD can also recruit FADD from the Fas receptor forming a TNFR1-TRADD-FADD complex, which induces apoptosis via caspase-8[57].

Apoptosis mediated via FADD appears to be regulated by cFLIP[55]. cFLIP inhibits the activation of caspase-8 and also promotes activation of NF-κB, thereby preventing receptor-mediated apoptosis.

Once apoptosis is initiated via procaspase-8 the release of cytochrome c from the mitochondria may occur, but is downstream from caspase activation and is not

required for receptor-mediated apoptotic cell death[56]. Active caspase-8 cleaves the Bcl-2 family protein, Bid, which then translocates to the mitochondria resulting in cytochrome c release and activation of the mitochondrial-mediated pathway. See Fig 3 for an illustration of receptor-mediated apoptosis.

With aging, TNF-α induced apoptosis has been shown to increase in T-cells[55] and endothelial cells[59] of aged humans. T-cells from aged humans are associated with decreased expression of TNF-R2 and TRAF2, which may be responsible for decreased NF-κB activation reported in aged T-cells, and increased expression of TNF-R1 and FADD, which may promote activation of caspase-8[55].

It is not known if TNF-α can activate apoptosis in aged skeletal muscle, despite reports of increased production of TNF-α by aged skeletal muscle[60]. It appears that TNF-α induces muscle atrophy via protein degradation rather than apoptosis in skeletal muscle from young animals[61]. The possibility of TNF-α induced apoptosis has not been investigated in skeletal muscle of aged animals. It may by possible that aging alters the expression of TNF-α receptors and adaptor proteins favoring caspase-8 activation and apoptosis rather than NF-κB activation, as found in aged T-cells[55]. This remains to be investigated.

Nuclear-mediated Apoptosis

Damage to nuclear DNA, such as double strand breaks, leads to the accumulation of p53 due to an increased half-life via phosphorylation. The accumulation of p53 induces either growth arrest to allow time for DNA repair mechanisms to repair the damage or, if the damage is too severe, p53 induces apoptosis. How p53 induces apoptosis is not yet clear, but evidence suggests that the

accumulation of p53 may alter the mitochondrial pathway resulting in cytochrome c release[62]. It is known that p53 increases the transcription of Bax and may also repress Bcl-2[63]. Schuler et al. demonstrated that overexpression of p53 induced cytochrome c release and caspase activation and that this was abolished by depletion of Bax[62]. Therefore, p53 may induce apoptosis via regulation of the Bcl-2 family proteins. However, other transcriptional targets of p53 are the Fas/Apo1 receptor and Death Receptor 5[63]. Ligand binding to these receptors activate procaspase-8 and procaspase-10, respectively, therefore increased availability of these receptors may lead to enhanced susceptibility to apoptosis. See Fig 3 for an illustration of nuclear-mediated apoptosis.

Inhibitors of Apoptosis

Several endogenous inhibitors of apoptosis have been identified such as inhibitor of apoptosis proteins (IAPs), apoptosis repressor with caspase recruitment domain (ARC), FADD-like interleukin-1-beta-converting enzyme inhibitory proteins (FLIPs), and heat shock protein 70 (HSP70) (Fig 2). The known IAPs include XIAP, cIAP1, and cIAP2. It is thought that these IAPs bind to cleaved/activated caspases and inhibit their activity[64]. For example, XIAP has been shown to bind to caspase-9 and inhibit the proteinase activity, thereby blocking apoptosis[65]. XIAP may also bind to caspase-3[64]. Another endogenous inhibitor of apoptosis is ARC which is only expressed in skeletal and cardiac muscle[66]. ARC has been shown to inhibit apoptosis by suppressing the activity of caspase-2 and caspase-8[66] and also can act by preventing cytochrome c release[67]. FLIP also inhibits the activity of caspase-8[68]. HSP70, a member of a family of protein chaperones induced by stress, can also act as

an apoptosis inhibitor by binding to Apaf-1 blocking the recruitment of procaspase-9 to the apoptosome[69].

Repressors of Inhibitors of Apoptosis Proteins

Recently a new mitochondrial protein, Smac/DIABLO, has been identified that acts as a repressor of IAPs (Fig 2). The Smac/DIABLO protein is released with cytochrome c from the mitochondria relieving the inhibition of caspase-9 by XIAP[64]. As another level of regulation of apoptosis, there may be a differential release of Smac/DIABLO and cytochrome c from the mitochondria[64]. This was suggested by the absence of Smac/DIABLO in S100 extract and the addition of detergent that is required for the release of Smac/DIABLO[64]. In conclusion, the release of Smac/DIABLO may then be required for those cell types that express IAPs.

Current Findings of Apoptosis and Skeletal Muscle

An increased rate of apoptosis with aging has been reported in several tissue types such as liver, brain, and heart[8,9], but has been ill investigated in aging skeletal muscle. However, an age-associated increase in the rate of apoptosis was reported in rhabdosphincter muscle cells of aged humans[18].

Apoptosis has been investigated under several pathophysiological conditions, such as muscular dystrophy[70], chronic heart failure[71], burn injury[72], and denervation[73], which all show an accelerated rate of skeletal muscle apoptosis. In addition, hindlimb unweighting (muscle disuse or unloading) showed significant apoptosis when assessed using nuclear DNA fragmentation[74]. Furthermore, acute exercise in untrained animals show significant evidence of apoptotic nuclei in skeletal muscle[70]. In summary, several reports have shown that skeletal muscle is capable of undergoing

apoptosis under pathophysiological conditions, but the extent of apoptosis in aged skeletal muscle and pathways which regulate apoptosis are not established.

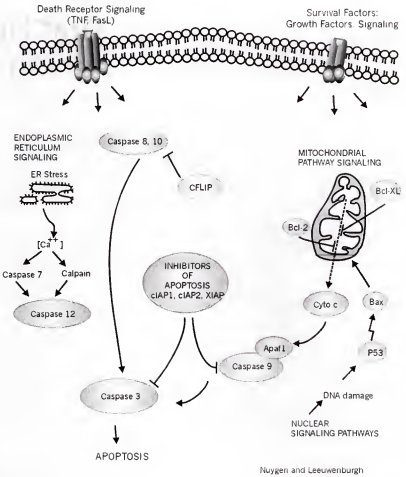

Fig 3. Simplified scheme of apoptotic pathways. A variety of pathways mediate apoptosis. The mitochondrial-mediated pathway may be stimulated by mitochondrial dysfunction followed by the release of cytochrome c. The endoplasmic-reticulum-mediated pathway may be activated via increased intracellular calcium content leading to the activation of procaspase-12. Receptor-mediated pathways may be initiated by ligand binding or the withdrawal of growth factors resulting in procaspase-8 activation. Lastly, the nuclear-mediated pathway may provoke apoptosis via the accumulation of p53 in response to extensive DNA damage.

Physiological stimuli inducing apoptosis in skeletal muscle are unknown. Most studies identifying myofiber apoptosis evaluated the effects of the

treatment/manipulation/disease on DNA fragmentation. The mechanisms (stimuli and signaling pathways) of apoptosis were not investigated. No research identifying physiological stimuli inducing apoptosis in skeletal muscle has been done. Three possible stimuli that may trigger apoptosis in aged skeletal muscle include mitochondrial dysfunction, ER stress and elevated intracellular calcium levels, and increased production of TNF-α. Therefore, the mitochondrial mediated, endoplasmic-reticulum-mediated, and receptor-mediated pathways were investigated in this study as possible contributing factors involved in the loss of skeletal muscle fibers with age.

A possible intervention to attenuate the loss of muscle cells and sarcopenia is caloric restriction. Caloric restriction has proven to be a powerful anti-aging intervention that attenuates the onset of age-related diseases and reduces the signs of aging.

Caloric Restriction

The anti-aging effects of caloric restriction were first described by McCay et al. in 1935[75]. Rats restricted in their food intake by 40% increase in their maximum life span approximately 30%. Since, numerous attempts have been made to elucidate the mechanisms contributing to the anti-aging effects of caloric restriction. Caloric restriction induces numerous biological changes, making it difficult to elucidate the mechanism(s) involved. The hypotheses that have obtained the strongest support are 1) decreased oxidative stress, 2) decreased plasma glucose and insulin, 3) hormesis, which is the ability to recruit defenses, and 4) alterations in gene expression [76, 77]. Another hypothesis is that a suppression of metabolic rate reduces oxidant production

and oxidative stress, thereby slowing the aging process. However, some investigators found that suppression of metabolic rate in caloric-restricted rats is transient. The metabolic rate adapts with time and is equivalent to that of control rats when normalized to fat free mass [77-80]. Caloric restriction has many beneficial effects on all or most organs, but I will focus on its effects on skeletal muscle.

The Effects of Caloric Restriction on Aged Skeletal Muscle

The effects of caloric restriction on aging skeletal muscle are extensive. Caloric restriction attenuates the age-dependent decrements in muscle function. Caloric restriction slows the rate of atrophy and fiber loss with age. Furthermore, caloric restriction attenuates mitochondrial dysfunction associated with aging as well as the accumulation of oxidative damage to proteins, lipids, and DNA. Lastly, aged skeletal muscle has been characterized by altered gene expression in which caloric restriction has been shown to reverse.

Caloric restriction (by 60%) was shown to increase the specific tension (kN/m^2) of the extentensor digitorum longus (EDL) and soleus muscle of 25-month-old rats as well as 32-month-old rats [16]. Specific tension is determined by several factors including excitation-contraction coupling (E-C coupling). E-C coupling diminishes with age and contributes to muscular weakness [81,82]. Two fundamental proteins involved in E-C coupling are the dihydropyridine receptor (DHPR), a voltage-sensitive Ca^{2+} channel located in the transverse tubules, and the ryanodine receptor (RyR), a calcium-activated Ca^{2+} channel located in the sarcoplasmic reticulum. As the action potential spreads along the sarcolemma and transverse tubules, the change in voltage causes a conformational change in the DHPR which in

turn induces a conformational change in the RyR resulting in calcium release from the SR. The content of DHPR and RyR are normally coupled in that their content is ~1:1 ratio, however with age this ratio may uncouple [83]. Mayhew et al. demonstrated that caloric restriction increased expression of the DHPR and RyR receptors in aged rats [16]. The improved coupling of these receptors may contribute to improved E-C coupling and increase specific tension in aged animals with caloric restriction.

Age-related muscular weakness can also be attributed to fiber atrophy and fiber loss with age, aside from alterations in E-C coupling. Absolute force production of a muscle is dependent on the physiological cross-sectional area of the muscle. Caloric-restricted animals have reduced muscle mass compared with their *ad libitum* counterparts, however the rate of muscle atrophy with age is diminished with caloric restriction [10]. Caloric-restricted animals also have attenuated loss of myofibers [5]. Caloric-restricted animals may therefore have a lower absolute muscle force production, but with age the loss in force is attenuated.

Tissue degeneration, atrophy, and cell death, may be mediated by mitochondrial dysfunction. Mitochondrial dysfunction in skeletal muscle increases with age but is attenuated in caloric-restricted rats[5]. The effects of caloric restriction on mitochondrial function have been assessed by measuring various Kreb's cycle and ETC enzyme activities such as succinate dehydrogenase (SDH) and cytochrome c oxidase (COX), however, ATP production has not been reported in the literature.

The increase in oxidative stress and the accumulation of oxidative damage to macromolecules within the mitochondria with age may cause mitochondrial dysfunction. Therefore, the attenuation of oxidative stress, mtDNA

deletions/mutations, and mitochondrial dysfunction with caloric restriction[5,14] may be due to a number of factors including decreased mitochondrial ROS production[84], increased DNA repair[85], enhanced proteolysis of damaged proteins and synthesis of new proteins[86], and enhanced antioxidant defenses[87,88], all which have been shown to occur with caloric restriction.

Gene expression is also altered with age and caloric restriction. Assessed by oligonucleotide microarray technology, aging of mouse gastrocnemius is characterized by lowered expression of proteins involved with protein turnover (synthesis and degradation), calcium homeostasis, and energy production[89]. Furthermore, aging increases expression of proteins that are induced by oxidative stress, DNA damage, muscle injury, and motoneuron degeneration. Caloric restriction reversed the majority of alterations that occurred with aging[89].

Caloric Restriction and Apoptosis

No reports have been published on the effects of caloric restriction on apoptosis in post-mitotic tissues, such as skeletal muscle. However, investigations have ensued on the effects of caloric restriction on mitotic tissues. Caloric restriction decreases the incidence of tumor formation and cancer[90,91]. An imbalance between proliferation and apoptosis in mitotic tissues may result in neoplasia or tumor formation. Therefore, a mechanism by which caloric restriction prevents cancer and tumor formation may be an increased rate of apoptosis. This would clear damaged or preneoplastic cells from the body. An increased rate of apoptosis has been reported in mitotic tissues such as liver and T-cells of caloric restricted animals compared with *ad libitum* counterparts[90,92]. These studies reported the change in the rate of apoptosis

but did not evaluate alterations in apoptotic proteins or signaling pathways that may have been involved. Future research will elucidate the mechanisms.

In summary, caloric restriction is a powerful anti-aging intervention. The mechanisms of its anti-aging effects are still to be elucidated but it is likely that the reduction in oxidative stress is intimately involved. The function of aged skeletal muscle is improved with caloric restriction, which could be attributed to enhanced E-C coupling and attenuation of fiber loss. Moreover, caloric restriction prevents mitochondrial dysfunction perhaps by decreasing oxidative stress within the mitochondria. Caloric restriction also reverses the alterations in gene expression that occur with aging. Lastly, the effects of caloric restriction on the rate of apoptosis and signaling pathways executing apoptosis in post-mitotic tissues are ill investigated.

Conclusion

Sarcopenia is in part due to loss of muscle cells with age. The role of apoptosis in age-related cell loss is unknown as well as apoptotic stimuli and signaling pathways that may be involved in the pathogenesis of sarcopenia. Mitochondrial dysfunction, elevated resting intracellular calcium levels, and increased TNF-α production may be stimuli commencing apoptosis via the mitochondrial mediated, endoplasmic-mediated, and receptor-mediated signaling pathways. Caloric restriction is an intervention that slows the aging process and may, therefore, prevent alterations in the rate of apoptosis and apoptotic signaling pathways that occur with aging in skeletal muscle.

CHAPTER 3
METHODS

Study 1

Animals

Male 6-month-old (n=8) and 24-month-old (n=8) Fischer 344 rats (National Institute of Aging colony, Harlan Sprague Dawley, Indianapolis, IN) were used. The rats were housed two per cage in a temperature- (18-22°C) and light-controlled environment with a 12-hour light/dark cycle, and were provided with food and water *ad libitum*. After one week of acclimation, the animals were randomly sacrificed on four consecutive days, with equal numbers of 6-month and 24-month old animals on each day.

Tissue Harvesting

Animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (5 mg/100 g body weight). The gastrocnemius muscle was excised and weighed. One section of the gastrocnemius was cut from the lateral head, frozen in liquid nitrogen, and stored at -80°C for further analysis while another section was cut from the medial head and used for mitochondrial isolation. The medial muscle section was homogenized in isolation buffer (0.225 M mannitol, 0.075 M sucrose, 0.2 % BSA, 1mM EDTA, pH 7.4) with a dilution of 1:25 (wt/vol) using a Potter-Elvehjem glass homogenizer, with the resulting homogenate centrifuged at 1,000g for 10 minutes. The supernatant was decanted and centrifuged at 14,000g for 10 minutes.

The 14,000g supernatant was decanted off and stored at -80°C for other biochemical analysis. The mitochondrial pellet was resuspended in 5 mL of wash buffer (0.225 M mannitol, 0.075 M sucrose, 1 mM EGTA, pH 7.4) and centrifuged at 14,000g for 10 minutes. The final mitochondrial pellet was resuspended in 0.5 mL storage buffer (0.25 M sucrose, 2 mM EDTA, pH 7.4) and mitochondrial membrane integrity was immediately determined. The remaining mitochondria were stored at -80°C for further analysis.

Functional Parameter

Determination of mitochondrial membrane integrity. We used two assays to evaluate if there were differences between the 6-month-old and 24-month-old rats in mitochondrial membrane integrity. Cytochrome c reduction in isolated intact mitochondria was determined immediately following the isolation procedure. Differences in membrane damage would result in higher levels of cytochrome c reduction by superoxide produced by the inner membrane. The incubation buffer consisted of 6 mM succinate, 70 mM sucrose, 220 mM mannitol, 2 mM Hepes, 25 mM KH_2PO_4, 2.5 mM $MgCl_2$, 0.5 mM EDTA, 5 μg/ml catalase, pH 7.4, and 40 μM acetylated cytochrome c. The change in absorbance was measured at 550 nm at 37°C using a spectrophotometric plate reader from Molecular Devices (Sunnyvale, CA). Furthermore, to determine if there were differences in mitochondrial membrane damage between the adult and old animals, we measured citrate synthase activity in the cytosolic and mitochondrial fractions using a previously described method [93]

Biochemical Assays

Determination of the levels of Bcl-2 and Bax by ELISAs. In order to quantify the amount of mitochondrial Bcl-2 and Bax proteins, ELISAs were performed. Plates were coated with 1 μg of mitochondrial protein in a physiological buffer solution (PBS) and sealed overnight at 4°C. Bcl-2 and Bax peptide standards (Oncogene, Boston) were included as positive controls. The plates were washed with buffer containing PBS with 0.02% sodium azide, and 0.05% Tween-20. The wells were blocked with 300μl of 1% BSA in PBS with 0.02% sodium azide and incubated at room temperature for 60 minutes. After washing of samples four more times, 50 μL of the primary antibody (Oncogene, Boston) at a concentration of 5 μg/mL diluted in 1% BSA in PBS/azide was incubated for 60 minutes at room temperature. Each well was washed four times. Next, 50μL of the secondary antibody (goat anti-rabbit IgG ALK-PHOS conjugate (Sigma A 8025) at a 1:2000 dilution into a solution of 1% BSA in PBS/azide) was added to each well and the plate was incubated for 60 minutes at room temperature. The washing procedure was then repeated and 100 μL of freshly made substrate containing para-nitrophenyl phosphate (Sigma N-2765) at a concentration of 1 mg/mL in substrate buffer (carbonate-bicarbonate, pH 9.6) was added. The plate was then incubated at room temperature for 60 minutes and the absorbance at 405 nm was read.

Determination of Apaf-1 by Western analysis. Apoptotic protease activating factor-1 (Apaf-1) level, an adaptor molecule essential for caspase-9 activation, was determined in the gastrocnemius muscle. Muscle was homogenized in phosphate buffer with a 1:10 (wt/vol) and centrifuged at 1,000g for 10 minutes.

Proteins were separated on a pre-cast 4-12% polyacrylamide gel (BMA, Rockland) using 60 μg of protein per well and then transferred onto a nitrocellulose membrane. Nitrocellulose membranes were blocked overnight using a blocking solution containing 0.05% Tween-20 and 5.0% milk. Membranes were incubated with the polyclonal antibody for Apaf-1 (dilution 1:500; Biovision, Palo Alto) for 90 minutes. Membranes were then incubated for 90 minutes in anti-rabbit Ig horseradish peroxidase (Amersham Life Science) diluted 1:1000. Blots were analyzed using the Apple-J program downloaded from the NIH website. Values are expressed as arbitrary OD units calculated by multiplying the area of each band by its optical density.

Apoptotic index. Apoptotic DNA fragmentation was quantified by measuring the amount of cytosolic mono- and oligo-nucleosomes using a Cell Death ELISA kit (Roche Molecular Biochemicals, Germany) according to instructions from the manufacturer. Endogenous endonucleases that cleave double stranded DNA in the linker region between nucleosomes generate mono- and oligo- nucleosomes of 180 base pairs or multiples[94]. This is a sensitive technique in quantifying apoptosis compared with (TUNEL) or DNA laddering, both being a qualitative assessment of DNA fragmentation. The assay is based on the quantitative sandwich-enzyme-immunoassay- principle. Wells are coated with a monoclonal anti-histone antibody. Nucleosomes in the sample bind to the antibody followed by the addition of anti-DNA-peroxidase, which reacts with the DNA associated with the histones. The amount of peroxidase retained in the immunocomplexes is determined photometrically with ABTS (2.2'-azino-di-[3-ethylbenzthiazoline sulfonate]) as a

substrate. All samples will be run in duplicate. Results will be reported as arbitrary OD units/ mg protein.

Cytosolic cytochrome c. Cytosolic cytochrome c was quantified using an ELISA kit (R&D Systems, Minneapolis) that employs the sandwich enzyme immunoassay technique.

Caspase-3 activity. Caspase activity was measured using the synthetic peptide n-acetyl-DEVD-AMC (BD PharMingen, San Diego). This assay detects activated caspase-3 and to a lesser extent caspases -6, -7, and -8. Active caspases will cleave the AMC from the peptide and the free AMC will fluoresce. Briefly, 1 mL of assay buffer (20mM HEPES, 10% glycerol, 2ul of 1 M DTT, and 14 µL of Ac-DEVD-AMC/mL of buffer) and 50 µL of sample were added to a microcentrifuge tube and protected from the light. Samples were incubated at 37°C for 60 minutes after which fluorescence was measured on a spectrofluorometer with an excitation wavelength of 380 nm and an emission wavelength of 440 nm. Protein concentration of mitochondria and cytosol were measured using the Bradford method[95].

Statistical Analyses

Analysis was performed in triplicate and the mean was used for statistical analysis. For statistical analysis, we used an independent t-test. Pearson correlation coefficients were determined using a Graph-pad Prism statistical analysis program (San Diego, CA). A p-value of < 0.05 was considered significant.

Study 2

Animals

Eleven 12-month-old *ad libitum* fed (AD12), eight 26-month-old *ad libitum* fed (AD26) and nine 26-month-old caloric restricted (CR26) male Fischer 344 rats (National Institute of Aging colony, Harlan Sprague Dawley, Indianapolis, IN) were used. Caloric restriction was started at 3.5 months of age (10% restriction), increased to 25% restriction at 3.75 months, and maintained at 40% restriction from 4 months throughout the animal's life. Fischer 344 rats were used as there is extensive background data available on these animals and they are the species we have used in previous projects. Males were used to avoid possible skeletal muscle protective effects of estrogen, which may function as an antioxidant. The rats were housed two per cage in a temperature (18-22°) and light-controlled environment with a 12-hour light/dark cycle. After one week of acclimation, the animals were randomly sacrificed (2 per day) on consecutive days.

Tissue Harvesting

Animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (5 mg/100 g body weight). The heart was excised. All of the hind limb muscles from both legs were then removed. Plantaris and gastrocnemius were weighed. Gastrocnemius muscle from the right leg was used for isolation of the mitochondrial and cytosolic fractions. The plantaris muscles was frozen in liquid nitrogen and stored at -80° for nuclear protein isolation.

Cellular Fractionation

Isolation of mitochondrial and cytosolic fractions. The right gastrocnemius muscle was homogenized in isolation buffer (0.225 M mannitol, 0.075 M sucrose, 0.2 % BSA, 1mM EDTA, pH 7.4) with a dilution of 1:25 using a Potter-Elvehjem glass homogenizer. Homogenate was centrifuged at 1,000g for 10 minutes. The supernatant was centrifuged at 14,000g for 10 minutes. The supernatant was subsequently stored at –80° for further analysis and used for measurements of parameters in the cytosolic proteins. The mitochondrial pellet was resuspended in 5 mL of isolation buffer and centrifuged at 14,000g for 10 minutes. The final mitochondrial pellet was resuspended in 0.5 mL of buffer.

Isolation of nuclear extracts. Nuclear extracts were isolated from plantaris muscle using the protocol described by Blough et al [96]. Briefly, 100 mg plantaris muscle was homogenized in 35 ml of Buffer 1 (10 mM HEPES, pH 7.5, 10mM $MgCl_2$, 5mM KCL, 0.1 mM EDTA, pH 8.0, 0.1% Triton X-100, 1 mM dithiothreitol, 0.1 MM phenylmethyl sulfonyl fluoride, 2 ug/ml aprotinin, and 2 ug/ml leupeptin) and centrifuged for 5 minutes at 3000g at 4°C. The pellet was then resuspended in 500 ul of Buffer 2 (20 mM HEPES,pH 7.9, 25% glycerol, 500 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, pH 8.0, 0.5 mM dithiothreitol, 0.2 mM phenylmethyl sulfonyl fluoride, 2 ug/ml aprotinin, and 2 ug/ml leupeptin). The sample was centrifuged for 5 minutes at 3000g at 4°C. The supernatant was transferred to a 5000 nominal molecular weight limit (NMWL), 4-ml Ultrafree Filter Unit (Millipore, Bedford, Ma) and centrifuged for 30 minutes at 4500g at 4°C. This last step concentrated the sample, which was then used for the measurement of AIF content.

Functional Assays

Oxygen consumption. To assess mitochondrial damage due to the isolation procedure we calculated the respiratory control ratio (state 3 respiration/state 4 respiration) which is commonly used as an index of the integrity of the mitochondrial preparation. Mitochondria respiration was monitored at 37°C by a biological oxygen monitor system (model YSI 5300, Yellow Spring Instruments, Columbus, OH) in incubation buffer (145 mM KCL, 30 mM Hepes, 5 mM KH_2PO_4, 3 mM $MgCl_2$, 0.1 mM EGTA, 0.1 % fatty-acid free albumin, pH 7.4), 2.5 mM pyruvate, 2.5 mM malate, and 0.25 mg mitochondrial protein for a total volume of 500 µl. State 4 respiration (no ADP) was read for 2 minutes and recorded using a chart recorder. State 3 respiration (with ADP) was measured in the presence of 500 µM ADP for 10 minutes or until the oxygen pressure is equal to zero. Oxygen consumption was calculated in ng O_2 consumed/minute/mg mitochondrial protein.

ATP production and content. We measured ATP production from isolated mitochondria using the bioluminescent technique for muscle tissue described by Wibom and Hultman[97] using a luminometer (model TD-20/20, Turner Designs, Sunnyvale). The assay utilizes D-luciferin which fluoresces in proportion to the presence of ATP and firefly luciferase. Freshly isolated mitochondria was added to a cuvette with ADP, Pi, metabolic substrates, and a Luciferin-Luciferase ATP monitoring reagent (Turner Designs, Sunnyvale). The metabolic substrate used was a mixture of 1 mM pyruvate and 1 mM malate. A blank cuvette containing no metabolic substrate was assayed to account for nonspecific ATP production. Known

ATP concentrations were used to establish a standard curve. Results are normalized to the control group (AD12), which is 100%.

Mitochondrial H_2O_2 production. The rate of mitochondrial H_2O_2 was measured using a fluorometric assay. Incubation buffer (145 mM KCl, 3 mM $MgCl_2$, 5 mM KH_2PO_4, 30 mM Hepes, 0.1 mM EGTA, 0.1% BSA, pH 7.4), mitochondria (0.25 mg/ml), horseradish peroxidase (5.7 U/ml), and homovanilic acid (0.1mM) were added to test tubes. The reaction was started by the addition of pyruvate and malate (2.5 mM each) and the tubes were placed in a shaking water bath for 15 minutes at 37°C. The reaction was stopped by placing tubes on ice and adding 0.5 ml of stop solution (0.1 M glycine, 25 mM EDTA, pH 12.0). Fluorescence was determined using a fluorescent microplate reader and a standard curve was generated for each analysis using glucose-glucose oxidase. Results are reported as nmol/min/mg mitochondrial protein.

Biochemical Assays

Cytosolic mono- and oligo-nucleosomes. DNA fragmentation was quantified by the amount of cytosolic mononucleosomes and oligonucleosomes using a Cell Death ELISA kit (Roche Molecular Biochemicals, Germany). Instructions from the manufacturer were followed using the cytosolic fraction of gastrocnemius muscle. Results were reported as arbitrary OD units/ mg protein. See Methods in Study 1.

Western blots. Proteins were separated on a 4-20% precast polyacrylamide gel (BMA, Rockland) and transferred onto a nitrocellulose membrane which was blocked overnight using a blocking solution containing 0.05% Tween and 5.0% milk.

Membranes were incubated for 90 minutes with the primary antibody at the desired dilution. Membranes were incubated for 90 minutes in anti-rabbit or anti-mouse Ig horseradish peroxidase (Amersham Life Science, United Kingdom) with an appropriate dilution. Blots were developed using ECL (Amersham Pharmacia Biotech, United Kingdom). The protein bands were analyzed using AppleJ program downloaded from the NIH website. Arbitrary OD units were calculated by multiplying the area of each band by its optical density and then normalized to the control group (AD12), which was 100%. Loading of equal amounts of protein was controlled for by using Ponceau staining (Pierce Biochemicals, Rockford, IL) of the nitrocellulose membrane.

Apaf-1 content. A Western blot was used to assess the content of Apaf-1. Whole gastrocnemius homogenate was used after a 10 minute centrifugation at 500xg. A polyclonal antibody for Apaf-1 was used (Biovision, Palo Alto).

AIF content. Total AIF content was measured using gastrocnemius homogenate and nuclear AIF content was measured using isolated nuclei. A Western blot protocol with a polyclonal AIF antibody (Alpha Diagnostic International, San Antonio) was used.

Cytosolic cytochrome c. Cytosolic cytochrome c was quantified using a cytochrome c ELISA kit (R&D Systems, Minneapolis, MN) which employs the sandwich enzyme immunoassay technique. Data is reported as nmol/mg cytosolic protein.

Caspase-9 content. Caspase-9 content was measured via Western blot using an anti-mouse monoclonal antibody (USBiological, Swampscott). Whole gastrocnemius homogenate was used after a 10 minute centrifugation at 500xg.

Caspase-3 content. Caspase-3 content was measured via Western blot using an anti-mouse monoclonal caspase-3 (BD Biosciences, USA). Whole gastrocnemius homogenate was used after a 10 minute centrifugation at 500xg.

Caspase-3 activity. Caspase-3 activity was measured using an activity assay kit (Roche Molecular Biochemicals) following the manufactures protocol. We used the cytosolic fraction of the gastrocnemius. This kit utilizes a caspase-3 antibody to isolate this caspase and a peptide substrate (DEVD-pNA), which is composed of a tetrapeptide and an attached chromophore. Caspase-3 will cleave off the chromophore after the recognized amino acid sequence DEVD and absorb light at 405 nm. A microplate was used for this assay and all samples were run in duplicate. Results are reported as arbitrary OD/mg protein.

NOTE: The only caspase enzyme activity measured was caspase-3 because this is the only caspase in which a commercial kit was available utilizing an antibody to first capture this caspase and therefore measures activity of the SPECIFIC caspase in question. All other commercial kits do not utilize a caspase specific antibody and assess "caspase-like" activity specific to a particular substrate.

Caspase-7 content. A Western blot was used to detect the full length protein and the (cleaved) activated caspase-7. Whole gastrocnemius homogenate was used after a 10 minute centrifugation at 500xg. A monoclonal anti-caspase-7 was used (US Biological, Swampscott).

Caspase-12 content. A Western blot was used to detect the full length and the activated (cleaved) caspase-12. Whole gastrocnemius homogenate was used after a 10 minute centrifugation at 500xg. A polyclonal (Ab-1) anti-caspase-12 antibody was used (Oncogene, Boston).

Caspase-8 content. A Western blot was used to detect the full length and the activated (cleaved) caspase-12. Whole gastrocnemius homogenate was used after a 10 minute centrifugation at 500xg. A polyclonal anti-caspase-8 antibody was used (Stressgen, Canada).

Inhibitors of apoptosis (XIAP, cFLIP, and ARC). Endogenous inhibitors of apoptosis were measured using Western blot. The cytosolic fraction was used to assess the content of these inhibitors. The following antibodies were used: monoclonal XIAP (MBL, Watertown), polyclonal ARC (Ab-1) (Imgenex, San Diego), antiserum cFLIP (Alexis, San Diego).

Protein concentration. Protein concentration was measured using the Bradford method[95].

Statistical Analyses

A t-test between groups (AD 12 vs AD26 and AD26 vs CR26) was used to analyze the data. Significance was established at $p<0.05$.

CHAPTER 4
RESULTS

Study 1

Morphological Measurements

Body weight and muscle mass of animals. Body weight increased ~20% in the 24-month-old rats (400 ± 8.3 g; mean ± SE) compared with the 6-month-old animals (319.0 ± 9.0 g; mean ± SEM). The gastrocnemius muscle mass showed a decrease between 6-months (1.3 ± 0.02 g; mean ± SEM) and 24-months (1.2 ± 0.02 g; mean ± SEM) of age. The loss in muscle mass is consistent with that of other studies[98,99]. In addition, expressed as a percentage of body weight, the gastrocnemius wet weight decreased significantly by 25% in the 24-month-old animals compared with 6-month-old animals (0.4±0.006 g vs. 0.3±0.006 g; mean ± SEM; $p<0.001$).

Mitochondrial Integrity

Membrane integrity was not different between the two groups due to the mitochondrial isolation procedure. To assure that mitochondria isolated from adult and old animals showed no differences in damage during the isolation procedure, we used two assays to determine membrane integrity (Table 1). One assay, to assess outer membrane integrity, involved measuring cytochrome c reduction by superoxide in intact isolated mitochondria. Greater levels in outer mitochondrial membrane damage would allow greater amounts of cytochrome c to enter the mitochondria and become reduced by superoxide produced by the inner membrane. In addition,

minimal damage to the inner (as well as the outer) mitochondrial membrane during the isolation procedure would result in a very low cytosolic citrate synthase activity compared with mitochondrial citrate synthase activity. We found that the cytosolic citrate synthase activity was approximately 5-7% of the total mitochondrial citrate synthase activity in both the 6- and 24-month-old animals (Table 1). Moreover, there were no significant differences in cytosolic or mitochondrial citrate synthase activity between the 6-month and 24-month-old rats.

Table 1. Mitochondrial membrane integrity in the gastrocnemius muscle of 6- and 24-month-old male Fischer-344 rats determined by the levels of cytochrome c reduction as well as mitochondrial and cytosolic citrate synthase activity.

	6-Month	24-Month
Cytochrome c reduction	0.08±0.01	0.08±0.04
Citrate synthase (mitochondrial)	2.0±0.15	2.1±0.30
Citrate synthase (cytosolic)	0.13±0.02	0.15±0.01

Results are expressed as mean ± SEM. Units of cytochrome c reduction are in arbitrary absorbance units at 550 nm/mg protein and citrate synthase units are in μmol/min/mg protein.

Biochemical Assays

Mono- and oligo-nucleosome content in muscle of 6-month and 24-month-old rats. Apoptosis results in the activation of endonucleases that cleave double stranded DNA between nucleosomes into 180 bp mono-nucleosome or multiple oligo-nucleosome fragments. We quantified the amount of DNA fragmentation in gastrocnemius muscle in the 6-month and 24-month-old animals. We found a 50% increase in cytosolic mono- and oligo- nucleosomes in the 24-month-old animals compared with the 6-month-old animals (p=0.0017), strongly suggesting an increase cell death by apoptosis (Fig 4).

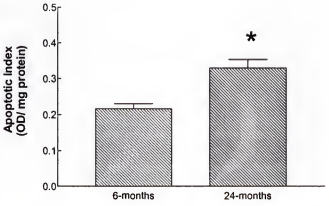

Fig 4. Apoptosis determined by the quantification of mono- and oligo- nucleosomes (apoptotic index) in gastrocnemius muscle of 6- and 24-month-old rats using an ELISA. The 24-month-old rats had significantly greater levels (+50%) of cytosolic mono- and oligo-nucleosomes compared with the 6-month-old rats (0.33 ± 0.02 vs. 0.22 ± 0.01, mean ± SEM; *p=0.0017). Results are reported as arbitrary OD units/mg protein.

Cytosolic cytochrome c and caspase-3 activity in skeletal muscle of 6-month and 24-month-old rats. Cytochrome c is a cofactor for procaspase-9 activation, which cleaves and activates procaspase-3 in the presence of Apaf-1 and initiates the caspase cascade. We did not detect a significant change in cytosolic

cytochrome c levels between the 6-month and 24-month-old animals (8.53 ± 2.44 vs. 9.91 ± 2.43 ng/mg protein, respectively; mean ± SEM; Fig 5A). Caspase-3, a pivotal protease involved in the destruction of the cell did not increase significantly in the 24-month-old rats compared with the 6-month-old animals (576 ± 60.3 vs 508 ± 43.4 arbitrary OD units/mg protein, respectively; mean ± SEM; Fig. 5B).

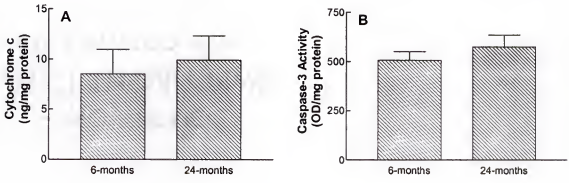

Fig 5. Cytosolic cytochrome c levels and caspase-3 activity in the gastrocnemius muscle in 6- and 24-month-old rats. (A) There was no significant difference in cytosolic cytochrome c between the 6- and 24-month-old animals (8.53 ± 2.44 vs. 9.91 ± 2.43, respectively; mean ± SEM). Results are reported as ng/mg protein. (B) Cytosolic caspase-3 activity was not significantly different between the 6- and 24-month-old animals (508 ± 43.4 vs. 576 ± 60.3, respectively; mean ± SEM; n=8 in each group). Caspase-3 activity is reported as arbitrary OD units/mg protein.

Significant correlation between cytosolic cytochrome c levels and the activity of caspase-3. We correlated cytosolic cytochrome c levels and caspase-3 activity to determine if the levels of cytochrome c present in the cytosol could directly affect the activity of caspase-3 (Fig 6). Indeed, we found a significant correlation ($r = 0.68$; $p = 0.0035$) between cytochrome c concentration and caspase-3 activity. However, when comparing within each age group, we only found a high correlation in cytochrome c and caspse-3 activity in the 6-month-old rats ($r = 0.79$; $p = 0.019$) but not in the 24-month-old rats ($r = 0.62$; $p = 0.102$). These findings strongly suggest

that cytosolic levels of cytochrome c correlate well with caspase-3 activities *in vivo* in 6-month old rats.

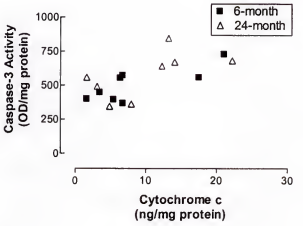

Fig 6. Correlation between cytosolic cytochrome c and caspase-3 activity in 6- and 24-month-old rats. The overall correlation for both 6-month and 24-month-old animals was ($r = 0.68$; $p = 0.0035$). We found a significant positive correlation ($r = 0.79$; $p = 0.019$) between cytochrome c and caspase-3 activity in the 6-month-old rats but did not reach significance in the 24-month-old rats ($r = 0.62$; $p = 0.102$).

Significant correlation between caspase-3 activity and mono- and oligo-nucleosome content. Since the activation of caspases are partly responsible for the formation of mono- and oligo-nucleosomes in that they cleave inhibitors of endonucleases, such as caspase activated DNase (CAD), we correlated caspase-3 activity with the amounts of mono- and oligo-nucleosome content in both age groups (Fig 7). We found no correlation ($r = -0.007$; ns) in the 6-month-old animals (Fig 7A), but a highly significant positive correlation in the 24-month-old rats ($r = 0.88$; $p = 0.001$; Fig 7B). Thus, the overall caspase-3 activity did not increase, but in those samples with a higher activity, the mono- and oligo-nucleosomes levels were elevated compared with other samples, suggesting a correlation.

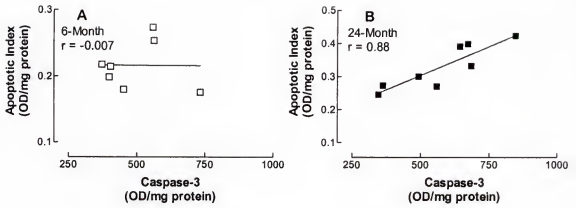

Fig 7. Correlation between caspase-3 activity and the apoptotic index in 6-month and 24-month-old rats. (A)We found no correlation ($r = -0.007$) between caspase-3 activity and the apoptotic index in the 6-month-old rats, (B) but a strong positive correlation in the 24-month-old rats ($r = 0.88$; $p = 0.0037$).

Detectable levels of Apaf-1 in rat skeletal muscle. Burgess et al. [100] showed that Apaf-1, a required apoptotic cofactor protein for procaspase-9 activation and therefore caspase-3 activation, was lacking in human skeletal muscle. We determined if Apaf-1 was present in skeletal muscle of rats. We found that Apaf-1 was detectable in muscle samples from adult and old rats (37550 ± 2291 vs 26440 ± 3580, respectively; n= 5; mean ± SEM of arbitrary OD units/mg protein) as determined by Western analysis (data not shown).

Mitochondrial Bcl-2 and Bax protein content in muscle of adult and old rats. We determined if there were any changes in the anti-apoptotic (Bcl-2) and pro-apoptotic (Bax) proteins in the mitochondria with age and determined the Bcl-2-to-Bax ratio (Fig 8). These Bcl-2 family proteins and others partly control the release of cytochrome c from the mitochondria. We found no significant alteration in Bcl-2 (Fig 8A) and Bax (Fig 8B) with age. In addition, the Bcl-2-to-Bax ratio in the mitochondria did not increase significantly (Fig 8C).

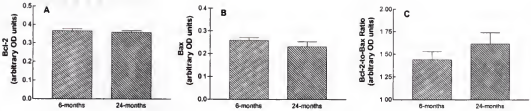

Fig 8. Mitochondrial Bcl-2, Bax, and the Bcl-2/Bax ratio, in gastrocnemius muscle of 6- and 24-month-old rats. Levels of the Bcl-2 family proteins were analyzed using an ELISA method (see Methods). (**A**) The mitochondrial Bcl-2 protein levels in the adult compared with the old rats were 0.37 ± 0.01 vs. 0.36 ± 0.01, respectively (mean ± SEM of arbitrary OD/mg protein). (**B**) The mitochondrial Bax protein levels in the adult compared with the old rats were 0.258 ± 0.01 vs. 0.231 ± 0.02 (mean ± SEM of arbitrary OD units/mg protein). (**C**) The gastrocnemius mitochondrial Bcl-2/Bax ratio in the adult compared with the old rats was 1.44 ± 0.09 vs. 1.62 ± 0.13.

Potential for cytosolic cytochrome c to activate the caspase cascade *in vitro*. Since we found a strong correletion between cytosolic cytochrome c and caspase-3 activity *in vivo*, we determined if it was indeed possible to activate the caspase cascade by the presence of cytosolic cytochrome c *in vitro*. Some tissue types contain caspase inhibitors blocking the activation of the mitochondrial-mediated pathway via cytochrome c release, therefore the caspase cascade is not activated by only the release of cytochrome c from the mitochondria. It is not known if this is the case in skeletal muscle. We determined if we could activate caspase-3 activity *in vitro* with the addition of cytochrome c. We incubated gastrocnemius muscle homogenates with 5 μg cytochrome c (from rat heart; Sigma, St. Louis) and ATP (1 mM; Sigma, St. Louis) or buffer (0.225 M mannitol, 0.075 M sucrose, 0.2% BSA, 1.5 mM $MgCl_2$, 1 mM EDTA) for 1 hour and measured caspase-3 activity (Fig 9). This concentration of cytochrome c has been previously used to activate caspase-3 activity in rabbit reticulocyte lysates[101].

We found no significant differences in caspase-3 activity in muscle homogenates of adult and old rats after incubation with exogenous cytochrome c. In contrast, liver homogenate from 6-month-old animals, used as a positive control, did show a ~50% increase in caspase-3 activity after cytochrome c incubation. Higher concentrations of cytochrome c (10 μg) gave the same results. Our results suggest that under these *in vitro* conditions, the caspase cascade cannot be activated by addition of cytochrome c to skeletal muscle homogenates.

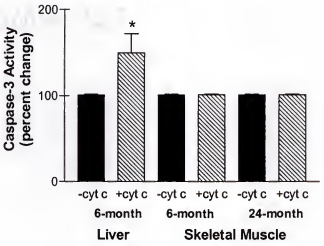

Fig 9. Cytochrome c addition *in vitro* was unable to activate procaspase-3 in gastrocnemius muscle of 6- and 24-month-old rats. Muscle and liver were homogenized 1:6 (weight/volume) in buffer (0.225 M mannitol, 0.075 M sucrose, 0.2% BSA, 1.5 mM $MgCl_2$, 1 mM EDTA, and 1 mM ATP). Homogenates were centrifuged at 1000g for 10 minutes. Five μg of cytochrome c (Sigma) or isolation buffer was added to 100 μl of cytosolic supernatant and incubated for 1 hour at 37°C followed by determination of caspase-3 activity (see Methods). Incubation with cytochrome c did not increase caspase-3 activity in muscle. However, caspase-3 activity was increased by ~50% in liver homogenate ($*p < 0.01$). The same results were obtained in two other independent experiments performed in triplicates.

We further investigated why caspase-3 activity did not increase with the addition of cytochrome c, suggesting that procaspase-9 was not being activated by cytochrome c or that procaspase-3 was unable to be activated by caspase-9. We

incubated muscle homogenate with recombinant active caspase-9 and found that caspase-3 activity increased in both the 6-and 24-month-old animals (Fig 10). These results indicate that activation of procaspase-9 may be the limiting step in the activation of the mitochondrial-mediated caspase cascade.

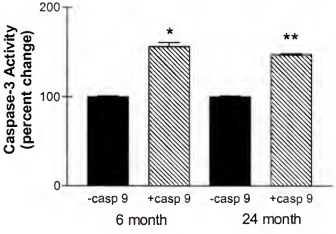

Fig 10. Recombinant active caspase-9 activates procaspase-3 *in vitro* in gastrocnemius of 6- and 24-month-old rats. Muscle was homogenized 1:6 (weight/volume) in buffer (0.225 M mannitol, 0.075 M sucrose, 0.2% BSA, 1.5 mM $MgCl_2$, 1 mM EDTA, and 1 mM ATP). Homogenates were centrifuged at 1000g for 10 minutes. Samples were incubated for 90 minutes at 37°C with or without 0.5 units of active human recombinant caspase-9 (one unit reflects the enzyme activity that cleaves 1 nmol of caspase substrate per hour at 37°C; US Biological, Massachusetts). Caspase-3 activity was determined using spectrophotometric techniques (see Methods). Incubation with active caspase-9 did increase caspase-3 activity in gastrocnemius of 6- and 24-month-old rats ($*p < 0.01$, $**p< 0.001$). To assure that caspase-9 was not directly cleaving the synthetic peptide, we incubated recombinant caspase-9 with the peptide in incubation buffer without tissue sample and found no significant cleaving of the synthetic peptide. The same results were obtained in two other independent trials performed in triplicates.

Study 2

Morphological Measurements

Body weight and muscle mass of animals. Body weight did not significantly change with age (410.7 ± 11.93 vs 384.4 ± 16.17 g, AD12 vs AD26), however caloric restriction resulted in a 31% reduction compared with AD26 (265.9 ± 6.29 g, CR 26, $p < 0.0001$). The gastrocnemius muscle mass declined with age (1.36 ± 0.03 vs 1.03 ± 0.05 g, $p < 0.0001$). Caloric restriction had no effect on muscle mass compared with AD26 (0.93 ± 0.05 g).

Functional Measurements

Mitochondrial membrane integrity. After isolating mitochondria, we determined the integrity of the outer mitochondria membrane by calculating the respiratory control ratio (RCR), the ratio between the state 3 and state 4 oxygen consumption. Using our isolation procedure, RCR ranged between 4-11 using pyruvate and malate as the substrates. These values are similar to those reported by others [102,103]. These results show that the mitochondria obtained were respiring well.

Mitochondrial oxygen consumption. State 4 respiration is the resting state of the mitochondrion when ADP is not present and is commonly used as an indication of the proton leak across the inner mitochondrial membrane. State 4 respiration increased by ~ 64% in the AD26 group compared with the AD12 group. The CR26 group decreased state 4 respiration compared with the AD26 group. These data suggest that the proton leak increases with age and that caloric restriction is able to prevent this change (Fig 11A).

State 3 respiration is the active ATP producing state of the mitochondrion when ADP is present. State 3 respiration was ~43% lower in the AD26 group compared with the AD12 group and caloric restriction did not appear to have an effect. These data suggest that the aging process suppresses state 3 respiration. Caloric restriction did not reverse this effect (Fig 11B).

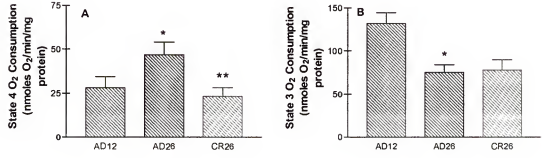

Fig 11. State 4 and state 3 mitochondrial oxygen consumption in adult and senescent rats fed *ad libitum* or caloric restricted. (A) State 4 respiration increased ~64% with age (28.17 ± 6.25 vs 46.76 ± 7.26 nmoles O_2/min/mg protein; *p = 0.04), while caloric restriction attenuated the age-related increase (23.28 ± 4.96 g, **p = 0.01). (B) State 3 respiration declined with age (132.1 ± 12.54 vs 75.13 ± 8.95 nmoles O_2/min/mg protein; *p = 0.003). Caloric restriction was not significantly different from AD26 (77.81 ± 11.99 nmoles O_2/min/mg protein).

Mitochondrial ATP production and ATP content. We assessed the maximal rate of ATP production by the mitochondria as well as the initial mitochondrial content of ATP. The data is normalized to the control group, which is AD12 (100%). ATP production declined ~60% with age. ATP production was similar between the AD26 and CR26 groups. These data suggest that the maximal ATP producing capacity of the mitochondria declines with age and caloric restriction did not seem to have an effect (Fig 12A).

Mitochondrial ATP content was reduced in the AD26 group compared with the AD12 group. ATP content was elevated in the CR26 group compared with the AD26 group. This data indicates that ATP content declines with age, which is attenuated by caloric restriction (Fig 12B).

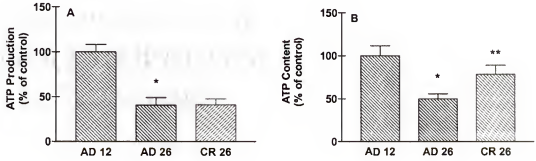

Fig 12. Mitochondrial ATP production and ATP content in adult and senescent rats fed *ad libitum* or caloric restricted. (A) Mitochondrial ATP production declines with age (100.0 ± 8.19 vs 40.8 ±8.43 %; $p < 0.0001$) and caloric restriction had no effect (41.2 ± 6.56 %). (B) Mitochondrial ATP content decreases with age (100.0 ± 11.67 vs 50.1 ± 5.65 %; *p = 0.004). Caloric restriction attenuated this decline (78.56 ± 10.61 %; **p = 0.02).

Mitochondrial hydrogen peroxide production. It has been shown that mitochondria in state 4 produce more hydrogen peroxide than mitochondria in state 3. Therefore, we measured state 4 mitochondrial hydrogen peroxide production. We did not detect an aging effect, however caloric restriction did reduce the hydrogen peroxide production compared with the AD26 group (Fig 13).

Biochemical Measurements

Apoptotic index. We measured cytosolic mono- and oligo- nucleosomes to determine the amount of apoptosis occurring in skeletal muscle at that time point of sacrifice. We found that aged skeletal muscle is associated with elevated levels of

mono- and oligo- nucleosomes. Caloric restriction attenuated this apoptotic marker as compared with AD26. These data indicate that the rate of apoptosis is elevated with age which is combated with caloric restriction (Fig 14).

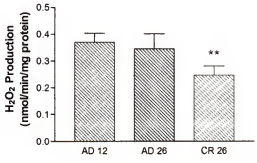

Fig 13. Mitochondrial hydrogen peroxide production in adult and senescent rats fed *ad libitum* or caloric restricted. Hydrogen peroxide production did not change with age (0.37 ± 0.03 vs 0.345 ± 0.06 nmol/min/mg protein), however it was lower in the CR26 group compared with the AD26 group (0.25 ± 0.03 nmol/min/mg protein; **p = 0.05).

Correlation between the apoptotic index and muscle weight. To determine the physiological consequence of the occurrence of apoptosis we report the correlation between the apoptotic index and the gastrocnemius muscle weight. We found that the correlation was highly significant suggesting that 60% of the variability in muscle weight may be due to apoptosis (Fig 15).

Cytosolic cytochrome c. Release of cytochrome c from the mitochondria into the cytosol is an initiating event for mitochondrial-mediated apoptosis. We first measured cytosolic cytochrome c via Western analysis and found that age nor caloric restriction had an effect on the content of cytosolic cytochrome c (Fig 16A). Since the variability was high, the analysis was repeated using an ELISA, a more sensitive

technique (Fig 16B). The ELISA results show a decrease in cytosolic cytochrome c with age while caloric restriction had no significant effect.

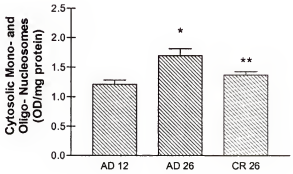

Fig 14. Apoptotic index in adult and senescent rats fed *ad libitum* or caloric restricted. Cytosolic mono- and oligo- nucleosomes were elevated in aged gastrocnemius (1.21 ± 0.07 vs 1.70 ± 0.12 arbitrary OD/mg protein; *p = 0.0008). Caloric restriction reduced the apoptotic index as compared with the AD26 group (1.37 ± 0.06 arbitrary OD/mg protein; **p = 0.01).

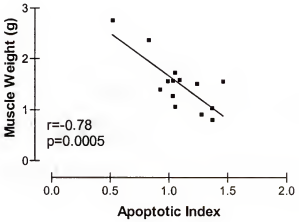

Fig 15. Correlation between the apoptotic index and gastrocnemius muscle weight in adult and senescent rats fed *ad libitum* or caloric restricted. As apoptotic index increases, the muscle weight decreases (r = -0.78; r^2 = 0.60; p = 0.0005; AD12 n = 5, AD26 n=4, CR26 n=5).

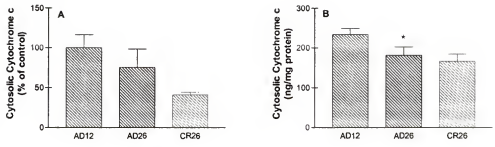

Fig 16. Cytosolic cytochrome c content measured by Western and ELISA analysis in adult and senescent rats fed *ad libitum* or caloric restricted. (A) Western blot analysis of cytosolic cytochrome c. Cytosolic cytochrome c did not change with age (100.0 ± 16.46 vs 75.60 ± 22.82 %; $p = 0.20$) or caloric restriction (41.20 ± 3.089 %; $p = 0.08$). (B) ELISA analysis of cytosolic cytochrome c. Cytosolic cytochrome c decreased with age (233.7 ± 15.58 vs 181.0 ± 21.29 ng cytochrome c/mg protein; $*p = 0.03$) and caloric restriction had no effect (165.8 ± 18.33 ng cytochrome c/mg protein).

ARC content and localization. ARC is an endogenous inhibitor that was first described to function as an inhibitor of caspase-2 and caspase-8 activation. Later, it was found that ARC also played a role in the mitochondrial-mediated pathway by preventing cytochrome c release from the mitochondria by an unknown mechanism. The total content of ARC did not change with age nor with caloric restriction (Fig 17A). However, we did find that the cytosolic levels of ARC tended to decline with age, although it was not significant, and that cytosolic ARC in muscle from caloric-restricted rats increased ~50% compared with the AD26 group (Fig 17B). Mitochondrial ARC increased with age while caloric restriction attenuated this effect (Fig 17C). These data suggest that ARC translocates from the cytosol to the mitochondrial membrane with age. Caloric restriction reduces the stimulus for ARC translocation to the mitochondria therefore reducing the mitochondrial content of ARC.

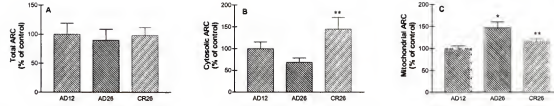

Fig 17. Total, cytosolic, and mitochondrial ARC content in adult and senescent rats fed *ad libitum* or caloric restricted. Total content of ARC did not change with age nor with caloric restriction (100.0 ± 6.20, 90.0 ± 18.91, and 98.0 ± 13.53 %). We found that the cytosolic levels of ARC tended to decline with age, although it was not significant (100.0 ± 15.70 vs 68.43 ± 10.43 %; p=0.07). However, cytosolic ARC in muscle from caloric-restricted rats increased ~50% compared with the AD26 group (145.3 ± 26.66 %; **p = 0.015). Mitochondrial ARC increased with age (100.0 ± 6.20 vs 147.3 ±13.17 %; *p = 0.008). Caloric restriction reduced mitochondrial ARC compared with AD26 (116.5 ± 6.24 %; **p = 0.03)

Caspase-9: zymogen and cleaved product. Apoptotic stimuli originating from the mitochondrion results in the activation of caspase-9. Cytochrome c, ATP and Apaf-1 with procaspase-9 (zymogen) form a complex resulting in an increase in the activity of caspase-9. We measured the content of the zymogen and the cleaved caspase-9. We show that the content of the caspase-9 zymogen is not affected by age or caloric restriction. The caspase-9 cleaved product tended to decreases with age (p=0.052), but was not affected by caloric restriction (Fig 18).

Apaf-1 content. Apaf-1 is a required cofactor for the activation of procaspase-9 and therefore an integral protein for the mitochondrial-mediated pathway of apoptosis. Apaf-1 increased with age while caloric restriction blunted this effect (Fig 19).

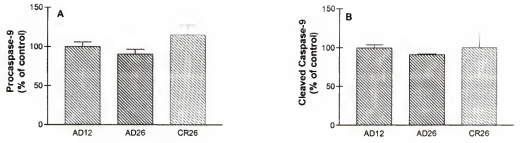

Fig 18. Caspase-9: zymogen and cleaved product in adult and senescent rats fed *ad libitum* or caloric restricted. (A) The content of the caspase-9 zymogen did not change with age or caloric restriction (100.0 ± 6.00, 90.5 ± 6.30, and 115.0 ± 12.39 %, respectively). (B) The content of the cleaved product of caspase-9 decreased 9% with age, although it did not reach significance (100.0 ± 4.0 vs 91.25 ± 0.63 %; p=0.052). Caloric restriction did not have an effect (100.2 ± 21.29 %).

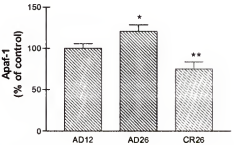

Fig 19. Apaf-1 content in adult and senescent rats fed *ad libitum* or caloric restricted. Apaf-1 increased with age (100.1 ± 5.70 vs 120.8 ± 7.76 %; *p = 0.03). Caloric restriction decreased Apaf-1 compared with AD26 (75.17 ± 8.40 %; **p = 0.001).

Caspase-3: zymogen, cleaved product, and enzyme activity. Procaspase-3 is the full-length inactive form of the cysteine protease, which is cleaved by an initiator caspase (such as caspase-9) to produce the active subunit. The procaspase-3 content increased with age and was significantly decreased with caloric restriction as compared with AD26 (Fig 20A).

The content of the active subunit of caspase-3 showed a similar trend as to the zymogen content. The content of the active subunit was elevated in aged gastrocnemius while the content declined in the CR26 group compared with AD26 (Fig 20B).

Despite the differences found with the zymogen and the active subunit between groups, there was no change in caspase-3 activity (Fig 20C). These results suggest that caspase-3 is being activated by an apoptotic stimulus, however, its activity is suppressed by an unknown mechanism.

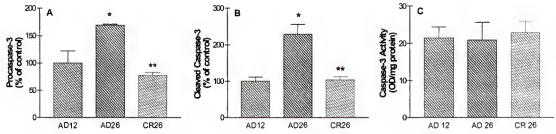

Fig 20. Caspase-3: zymogen, cleaved product, and enzyme activity in adult and senescent rats fed *ad libitum* or caloric restricted. (A) The procaspase-3 content increased with age (100.0 ± 22.27 vs 169.0 ± 1.87 %; *p = 0.015) and was significantly decreased with caloric restriction as compared with AD26 (77.2 ± 5.45 %; **p < 0.0001). (B) The content of the active subunit was elevated in aged gastrocnemius (100.0 ± 11.67 vs 229.0 ± 27.37 %; *p = 0.0001) while the content declined in the CR26 (103.8 ± 8.24 %; **p < 0.0001) group compared with AD26. (C) There was no difference in caspase-3 activity between groups (21.47 ± 2.92, 20.90 ± 4.69, and 22.88 ± 2.94 arbitrary OD/mg protein; AD12, AD26, and CR26, respectively).

Content of XIAP. XIAP is an endogenous inhibitor of apoptosis that binds to active caspase-3 and inhibits its protease activity. We measured the content of XIAP because the presence of this inhibitor may be responsible for the suppression of caspase-3 activity in skeletal muscle despite cleavage of the zymogen. The content of

XIAP was elevated in aged skeletal muscle and reduced with caloric restriction compared with AD26 (Fig 21). These results follow the same trend as those for the content of the active caspase-3 subunit. Therefore, these data may suggest that XIAP is elevated in aged skeletal muscle to combat the rise in the active caspase-3 content to assure inhibition of protease activity.

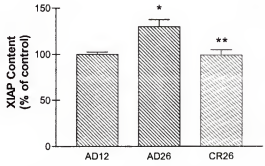

Fig 21. Content of XIAP in adult and senescent rats fed *ad libitum* or caloric restricted. The content of XIAP was elevated in aged skeletal muscle (100.0 ± 2.45 vs 129.9 ± 7.65 %; *p = 0.0015) and reduced with caloric restriction compared with AD26 (99.1 ± 5.73 %; **p = 0.0045).

AIF: total and nuclear content. AIF translocation to the nucleus can induce DNA fragmentation in a caspase-independent manner. We measured total AIF content as well as nuclear content in gastrocnemius. Total AIF (Fig 22A) was shown to increase with age. Lifelong caloric restriction resulted in a reduction of total AIF compared with the AD26 group. Nuclear content of AIF was not altered by age or caloric restriction (Fig 22B).

Caspase-12: zymogen & cleaved product. Caspase-12 is associated with the endoplasmic reticulum and is activated by a disruption in calcium homeostasis. We detected a striking 350% increase in the content of procaspase-12 in aged

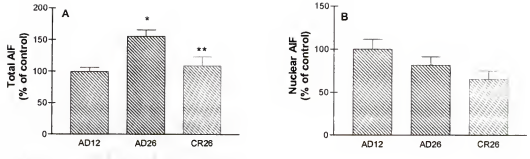

Fig 22. Content of total and nuclear AIF in adult and senescent rats fed *ad libitum* or caloric restricted. (A) Total AIF was shown to increase with age (100.0 ± 6.7 vs 155.5 ± 10.5 %; *p = 0.002). Caloric restriction resulted in a reduction of total AIF compared with the AD26 group (109.2 ± 14.35 %; **p = 0.015). (B) Nuclear content did not change with age or with caloric restriction (100 ± 11.42, 81.5 ± 9.75, and 65.25 ± 9.59 %).

gastrocnemius muscle. We also show that caloric restriction reduces this age-related increase by ~48% (Fig 23A). These data may suggest an increased demand for the caspase-12 protease in aged skeletal muscle.

The active subunit of the cleaved product did not change with age but decreased with caloric restriction compared with AD26 (Fig 23B). The reduced presence of the active subunit may play a part in reducing the apoptotic index found in skeletal muscle of caloric-restricted animals.

Caspase-7: zymogen & cleaved product. During ER stress caspase-12 is activated via caspase-7. Therefore, we assessed the content of the caspase-7 zymogen and the activated product. We did not detect a change in procaspase-7 between groups (Fig 24A). However, we do show that caloric restriction decreases the content of the activated product of caspase-7 by ~30% compared with the AD26 group (Fig 24B).

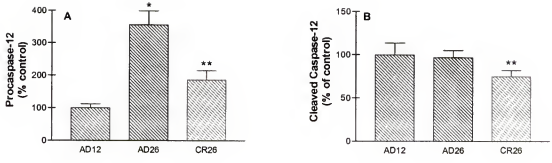

Fig 23. Caspase 12: zymogen and cleaved product in adult and senescent rats fed *ad libitum* or caloric restricted. (A) Procaspase-12 expression increased dramatically with age (100.0 ±12.29 vs 356.4 ± 43.10 %; *p = 0.0002). Caloric restriction reduced procaspase-12 content compared with the AD26 group (186.0 ± 29.64 %; **p = 0.006. (B) The active subunit of the cleaved product did not change with age but decreased with caloric restriction (100.0 ± 13.61, 96.56 ± 8.51, and 74.46 ± 7.23 %; AD12, AD26, and CR26, respectively; **p = 0.04).

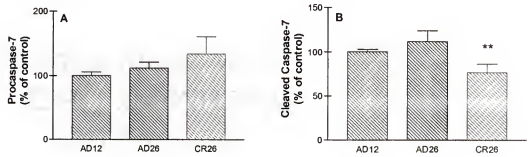

Fig 24. Caspase-7: zymogen and cleaved product in adult and senescent rats fed *ad libitum* or caloric restricted. (A) Procaspase-7 does not change with age or caloric restriction (100.2 ± 5.51, 112.0 ± 9.51, and 134.2 ± 27.13 %, respectively). (B) The content of the cleaved product does not change with age (100.2 ± 2.60 vs 111.8 ± 12.07 %), although caloric restriction decreased the content as compared with AD26 (76.54 ± 9.74 %; **p = 0.026).

Caspase-8: zymogen and cleaved product. Caspase-8 is involved with the receptor-mediated pathway of apoptosis. The expression of procaspase-8 (Fig 25A)

was not different between groups nor was the presence of the active subunit of the cleaved product (Fig 25B). These data suggest that this pathway may not contribute to age-related apoptosis in the gastrocnemius.

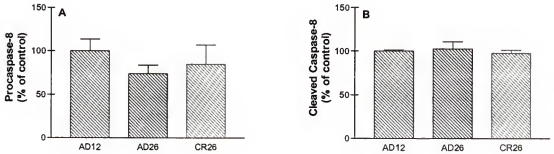

Fig 25. Caspase-8: zymogen and cleaved product in adult and senescent rats fed *ad libitum* or caloric restricted. (A) The expression of procaspase-8 (100.0 ± 13.27, 74.2 ± 9.55, and 85.0 ± 22.08 %; AD12, AD26, and CR26, respectively; Fig 11A) was not different between groups. (B) The content of the active subunit of the cleaved product did not change with age or caloric restriction (100.0 ± 1.41, 102.5 ± 8.34, and 97.4 ± 3.57 %; AD12, AD26, and CR26, respectively).

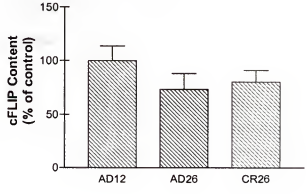

Fig 26. Content of cFLIP in adult and senescent rats fed *ad libitum* or caloric restricted. The content of cFLIP was not altered by age or caloric restriction (100.0 ± 13.56, 73.43 ± 14.49, and 80.15 ± 10.88 %; AD12, AD26, and CR26, respectively).

Content of cFLIP. cFLIP is an endogenous inhibitor that prevents the activation of procaspase-8 to its active form. The content of cFLIP was not altered by age or caloric restriction (Fig 26).

CHAPTER 5
DISCUSSION

Study 1

Apoptosis and Aging

An increased rate of apoptosis in skeletal muscle has been documented to occur under several pathophysiological conditions[70,73,74,104]. However, there is little evidence and investigation as to whether or not apoptosis occurs in post-mitotic tissues during normal physiological aging. We were able to detect a significant increase in apoptosis (mono- and oligo-nucleosomes) in skeletal muscle from aged animals. This supports the recent findings of Strasser et al.[18] who found an increased incidence of apoptosis by the terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) technique in human rhabosphincter skeletal muscle with age. Our results provide the first report to show a significant increase in apoptosis in aged locomotor skeletal muscle of rodents.

To elucidate possible pathways of apoptosis, which may have lead to the cleavage of DNA to form mono- and oligo- nucleosomes in aged muscle, we evaluated the levels of cytochrome c and caspase-3 activity in the cytosol of young and old rats. It has been shown that using a model of acute skeletal muscle burn injury results in an increase in cytosolic cytochrome c, caspase-3 activity, and apoptosis[72]. However, with aging skeletal muscle, a slow chronic process, we did not find significant increases in cytosolic cytochrome c or caspase-3 activity. Further analysis revealed a significant positive correlation between the levels of cytosolic

cytochrome c and caspase-3 activity. These findings may suggest that a few fibers may be undergoing apoptosis at a given time point making it difficult to detect. However, we were able to pick up the more subtle correlation between cytosolic cytochrome c and caspase-3 activity. Therefore, it cannot be entirely ruled out that mitochondrial-mediated apoptosis does not occur with aging in muscle.

Another important finding was the positive correlation between caspase-3 activity and mono- and oligo- nucleosomes in the 24-month-old animals, but not in the 6-month-old animals. It is well established that caspase-3 is able to cleave endonuclease inhibitors and therefore activate caspase-dependent DNase (CAD), which is responsible for the formation of mono- and oligo-nucleosomes[105]. Therefore, this strongly suggests that caspase-3 activity in the old rats may be responsible for the activation of CAD and the increase in DNA fragmentation seen in the old rats. In addition, caspase-independent DNA fragmentation has been shown to occur via release of apoptosis inducing factor (AIF) from the mitochondria and translocation to the nucleus[48] and may also play a role in DNA fragmentation of young and old animals, a possibility that requires further investigation.

The Bcl-2 family of proteins partly regulates the release of cytochrome c from the mitochondria. The mechanism by which this occurs is not yet clear, however, it is thought that the ratio between Bcl-2-to-Bax may be one determining factor influencing cytochrome c release[43]. The Bcl-2-to-Bax ratio was slightly increased, but this change was not significant and therefore cannot explain why there was little release of cytochrome c from the mitochondria in skeletal muscle with aging. We should point out that our isolation technique only isolates sub-sarcolemmal

mitochondria from skeletal muscle. It may be possible that age-related alterations in mitochondrial Bcl-2 and Bax may occur in the interfibrillar fraction of mitochondria rather than the sub-sarcolemmal fraction. Future investigations will clarify the role of these mitochondrial sub-populations in apoptosis. Many other Bcl-2 family proteins, such as Bak, Bcl-X_L, Bid, Bad, Mcl-1 can influence the release of cytochrome c and may also play a role in skeletal muscle during aging. Moreover, once cytochrome c is released, inhibitors of caspases, or even repressors of these inhibitors could play a role in regulating the activation of caspases by cytochrome c. Specifically, IAPs can inhibit caspases while second mitochondrial-derived caspase activators (SMAC) can repress some of these inhibitors[66,106].

We have attempted to elucidate the regulation of the mitochondrial-mediated signaling pathway in skeletal muscle. In cell types, such as liver, the addition of cytochrome c to cytosol activates caspase-3 *in vitro*[107]. We demonstrated that the caspase cascade (as detected by procaspase-3 activation) in skeletal muscle was not activated in response to addition of cytosolic cytochrome c. Our findings are in agreement with Burgess et al.[100] who reported that the addition of cytochrome c to human skeletal muscle cytosol did not increase caspase-3 activity. However, they suggested that this was due to a lack of Apaf-1, a protein required for the activation of procaspase-9. In contrast, we found significant levels of Apaf-1 in rat skeletal muscle and thus suggests that the inability of cytosolic cytochrome c to activate procaspase-9 in rat skeletal muscle was not due to the lack of this cofactor.

Additional experiments were performed to determine why increased cytosolic cytochrome c *in vitro* did not result in an increase in caspase-3 activity. We

demonstrated that procaspase-3 was indeed capable of being activated by active caspase-9 and, therefore, it appears that the activation of procaspase-9 may be the limiting step in the activation of the caspase cascade via mitochondrial cytochrome c release.

Conclusion

This study is the first to explore the role of apoptosis in sarcopenia and suggests that subtle changes in apoptosis are involved, which may be critical over a longer period of time. Our results suggest that aged skeletal muscle is characterized by an increased rate of cell death, however, it is not clear if mitochondrial-mediated pathways are prevalent in causing apoptosis in skeletal muscle, but cannot be entirely excluded at this point. Other apoptotic pathways may also significantly contribute to cell death, such as the receptor-mediated pathway via TNF-α. A recent study showed a significant increase in TNF-α protein expression with age in human skeletal muscle[60]. Further investigation will be required to elucidate the involvement of this pathway contributing to skeletal muscle fiber loss via apoptosis.

Furthermore, since whole muscle tissue was used to quantify apoptosis, the possibility remains that other cell types, such as connective tissue, endothelial tissue, and nervous tissue contributed to the apoptosis observed. However, we removed connective tissues and nervous tissue during the dissection of muscle tissues and therefore the majority of cells used in this study were myocytes.

Finally, our data suggest that skeletal muscle is not a tissue that initiates apoptotic signaling cascades in response to cytosolic cytochrome c alone. It appears that the activation of the caspase cascade mediated by mitochondrial cytochrome c

release may be limited by the activation of procaspase-9 suggesting that this caspase may be associated with an inhibitor of some type. Alternatively, other proteins, such as heat shock protein 70 could bind to Apaf-1 and inhibit the recruitment of procaspase-9 to the apoptosome, thereby inhibiting apoptosis[69] and may function as a protective mechanism against muscle fiber loss. These possibilities remain to be investigated.

Study 2

Caloric restriction is a powerful anti-aging intervention. Among its life extending effects is the attenuation of skeletal muscle fiber loss, which is known to contribute to sarcopenia during old age. Therefore, we used caloric restriction as an intervention to slow the aging process to give us more insight into the role of apoptosis in aging muscle. The mechanism by which caloric restriction slows the aging and death of muscle fibers is not yet clear. However, strong evidence supports the hypothesis that oxidant production and mitochondrial dysfunction caused from free radical damage is reduced with caloric restriction and consequentially slows the aging process. Mitochondrial dysfunction is a stimulus for apoptosis *in vivo*[37], therefore, since caloric restriction reduces this apoptotic stimulus we investigated the effect on the occurrence of apoptosis and apoptotic signaling pathways in skeletal muscle.

Caloric Restriction and Mitochondrial Function

We found that caloric restriction reduced mitochondrial hydrogen peroxide production in skeletal muscle. These results are consistent with mitochondrial oxidant production in other tissues such as liver, brain, and heart of caloric restricted

animals[108,109], but we are the first to show data describing the effects of caloric restriction on mitochondrial hydrogen peroxide production in skeletal muscle. We did not see an increase in H_2O_2 production with age as shown by Sohal et al., in house fly skeletal muscle[21]. These differences may have been attributed to use of an alternative substrate, alpha-glycerophosphate compared to pyruvate and malate, which we used[21]. Bejma et al., also found an increase in mitochondrial oxidant production in rat skeletal muscle with age but the assay used did not distinguish between reactive oxygen species or reactive nitrogen species production[4], thereby making it difficult to compare results. Lastly, superoxide anion production was shown to increase with age in submitochondrial particles from mouse skeletal muscle[14]. However, oxidant production from submitochondrial particles is not comparable to intact mitochondria as used in our procedure. To our knowledge, these are the only three papers published measuring mitochondrial oxidant production in aged skeletal muscle.

State 4 oxygen consumption gives an indication of the proton leak across the inner mitochondrial membrane and this has been shown to increase with age[102]. We show that lifelong caloric restriction prevents the age-associated increase in state 4 respiration suggesting that caloric restriction prevents an increase in the proton leak. These data agree with those of Weindruch's group[102]. The mechanism by which the proton leak increases with age, or by which caloric restriction reduces the proton leak, is not known but a few possibilities exist. First, oxidative damage to proteins and lipids of the mitochondrial membrane may allow for a leakier membrane. Second, aged skeletal muscle has been shown to contain higher levels of uncoupling protein 2

(UCP2) resulting in an increased proton leak[110]. Lastly, induction of the mitochondrial permeability transition pore would provide enhanced permeability across the outer membrane allowing for leakage of protons out of the intramembrane space[111]. Caloric restriction may affect one or a combination of these mechanisms. The increased proton leak with age comes at the expense of ATP production resulting in a decline of ATP content available for cellular function.

As expected mitochondrial ATP production and ATP content declined with age[112]. Although caloric restriction did not attenuate the suppressed ATP production it did have an effect on the mitochondrial ATP content. There are no published data measuring ATP production and ATP content after lifelong caloric restriction, so we cannot compare our results. However, caloric restriction has been shown to attenuate damage to mitochondrial enzymes reducing the number of fibers containing the cytochrome c oxidase deficient (COX^-) and succinate dehydrogenase hypersensitive (SDH^{++}) phenotype in mice and rats[5,17]. Caloric restriction also reduces the amount of mtDNA deletions that accumulate with age, which is thought to contribute to electron transport chain (ETC) abnormalities[17]. Thus, we were surprised to find that caloric restriction did not improve ATP production.

Our findings suggest that caloric restriction may reduce metabolic rate in skeletal muscle. These results are consistent with Rumsey et al.[113], which is the only other study that measured state 3 oxygen consumption in skeletal muscle of caloric-restricted rats. However, several studies have found no difference in metabolic rate normalized to lean body mass between *ad libitum* fed and caloric-restricted rats[78-80,114]. Greenberg and Boozer state that these studies normalizing metabolic rate to

lean body mass using calculations of fat-free mass, body weight, body weight$^{0.75}$, or body weight$^{0.67}$ [78-80,114] may not be using a reliable estimate of metabolic mass and, therefore, need to reevaluate the role of suppressed metabolic rate as a mechanism contributing to the life extending properties of caloric restriction[115]. They show that the best estimate of metabolic mass was the combined mass of of the heart, liver, kidneys, and brain[115]. It seems possible that individual organs of the body may respond differently to caloric restriction. For example, the metabolic rate of skeletal muscle may decrease with lifelong caloric restriction as shown by Rumsey et al. and ourselves, while that of other organs, such as liver, may increase with lifelong caloric restriction[113].

In summary, caloric restriction reverses age-related mitochondrial dysfunction, reducing an apoptotic stimulus. Caloric restriction suppresses mitochondrial oxidant production, reduces state 4 oxygen consumption, and has been shown by others to prevent oxidative damage to mtDNA and proteins of the ETC. Although ATP production remains suppressed with caloric restriction due to a reduced metabolic rate, this rate of production seems sufficient to supply the required energy for the muscle cells to maintain homeostasis and to prevent cellular degeneration as seen with normal aging.

Aging, Caloric Restriction, and Apoptosis

Apoptosis is strongly correlated with the loss of muscle mass with age and therefore may be an important mechanism contributing to sarcopenia. Caloric restriction attenuates the loss of muscle fibers with age, therefore we assessed an apoptotic index, mono- and oligo- nucleosomes, to determine if caloric restriction

acts by slowing the rate of apoptosis. We show that caloric restriction reduces the apoptotic index in aged skeletal muscle. This study is the first to investigate the effects of caloric restriction on apoptosis and essential apoptotic proteins in skeletal muscle or any other post-mitotic tissue for that matter.

I will first discuss the effects of age on the various apoptotic pathways starting with the mitochondrial-mediated pathway, followed by the endoplasmic-reticulum-mediated, and receptor-mediated pathways. I will finally discuss the effects of caloric restriction.

Age-related alterations in apoptotic pathways. First we investigated age-related alterations in the mitochondrial-mediated pathway. We assessed cytosolic cytochrome c content as an indication of cytochrome c release by the mitochondria *in vivo*. Cytosolic cytochrome c content did not increase with age as we hypothesized, but instead decreased. We attribute this to an increase in protective mechanisms against cytochrome c release. Several adaptations are possible. First, the apoptosis inhibitor ARC regulates cytochrome c release from the mitochondria. In conditions of mitochondrial stress, it has been shown that ARC translocates from the cytosol to the mitochondrial membrane and prevents cytochrome c release and apoptosis[67,116]. With aging, we show that cytosolic ARC tended to decrease ~30% ($p=0.07$), while mitochondrial ARC increased significantly. Since total ARC remained unchanged with age, our data suggests that aging results in the translocation of ARC from the cytosol to the mitochondrial membrane preventing cytochrome c release. Secondly, adaptations in the Bcl-2 family proteins could protect against cytochrome c release if resulting in an increase in the ratio between anti-apoptotic verses pro-apoptotic Bcl-2

proteins. In our previous study[117] we did not find a change in mitochondrial Bcl-2 or Bax content, but there was a trend for an increase in the Bcl-2/Bax ratio (p=0.10). This adaptation would protect against cytochrome c release from the mitochondria. We do not know the aging effects on other Bcl-2 family proteins in skeletal muscle since no such studies exist in the literature. However, age-associated alterations in the mitochondrial Bcl-2/Bax ratio in other tissues have been reported and seem to be tissue specific. The Bcl-2/Bax ratio was shown to increase in mitochondria from rat brain cortex due to a significant decrease in Bax content with age (data from our laboratory, in press). In contrast, mitochondria from aged rat heart showed a strong trend for a decrease in the Bcl-2/Bax ratio due to a decline in Bcl-2 with age, which may contribute to the increased cytochrome c release as shown[118]. Thirdly, some proteins making up the mitochondrial permeability transition pore are redox sensitive, including one in equilibrium with mitochondrial glutathione[119]. Leeuwenburgh et al., has shown that glutathione is increased in skeletal muscle with age[99] and may therefore play an important role in preventing the activation of the permeability transition pore opening and cytochrome c release.

Since cytosolic cytochrome c did not increase with age, we did not expect caspase-9 activation to increase since cytochrome c is a stimulus and required cofactor for its activation. We have shown that neither age nor caloric restriction affected caspase-9 activation *in vivo*.

Caspase-3 is the central executioner of apoptosis and is activated/cleaved via many "initiator" caspases, such as caspase-8, -10, and -2, as well as caspase-9. Caspase-3 cleavage increased with age, however, the enzyme activity did not. This

discrepancy may be due to the presence of endogenous inhibitors of apoptosis, which can bind to active caspase-3 and inhibit its activity. We did find that XIAP content was elevated in aged muscle tissue and we speculate that this increase may inhibit the activity of the elevated content of cleaved caspase-3 observed in aging muscle. The expression of IAPs is under the control of the transcription factor NF-κB[120], which is activated by intracellular oxidants and inflammatory cytokines, among numerous other stimuli, resulting in the transcription of many anti-apoptotic and pro-survival proteins[121,122].

To conjecture on the role of the mitochondrial-mediated pathway in aging, it appears that the occurrence of apoptosis in aged skeletal muscle may not be mediated via mitochondrial cytochrome c release as hypothesized. The mitochondria appear to adapt to protect against apoptosis and the persistent expression of XIAP may be blocking activity of caspase-3. We look for alternative pathways involved with apoptosis in aged skeletal muscle.

Apoptotic stimuli can also result in the release of AIF from the mitochondria. AIF translocates to the nucleus where it induces large scale DNA fragmentation via a caspase-independent mechanism. We measured total and nuclear content of AIF to assess its role in age-related apoptosis. We show that total AIF content increased with age, however nuclear content remained unchanged. As discussed, adaptations with age may prevent mitochondrial release of apoptotic proteins, including AIF. AIF is flavoprotein that can also act as a mitochondrial oxidoreductase, aside from its role in apoptotic DNA fragmentation[123]. Flavoproteins can act as ROS/RNS scavengers and prevent lipid peroxidation, therefore an increased total content of AIF

with age may be in response to enhanced oxidative stress which is seen in aging skeletal muscle.

Caspase-12 is involved with apoptosis induced by loss of calcium homeostasis and ER stress. We found a ~350% increase in the expression of the caspase-12 zymogen in aged gastrocnemius. Rao, et al., showed that induction of ER stress was a specific signal to induce expression of caspase-12[53]. Therefore, our results suggest that aged skeletal muscle is characterized by severe ER stress. The causes of age-related ER stress and calcium dyshomeostasis in skeletal muscle are not clear. Oxidative stress within the cell, which could stem from oxidant release from the mitochondria, causes damage to proteins of the ER membrane such as the Ca^{++} ATPase that pumps cytosolic calcium back into the ER[50,51]. Moreover, suppressed ATP production by the mitochondria may result in lower activity of these calcium pumps making it difficult to maintain low levels of cytosolic calcium[124]. Another possible mechanism for calcium dyshomeostasis may be the inability of the mitochondria to sequester cytosolic calcium due to age-related mitochondrial dysfunction. Despite the profound increase in the zymogen, age did not alter the content of the activated form of caspase-12. It is possible that the cleaved product is unstable and therefore may be difficult to detect increased content[125]. Very little is known about the mechanism of caspase-12 induced apoptosis. Future research will elucidate its downstream substrates leading to apoptosis and its role in the aging process.

Tumor necrosis factor alpha (TNF-α) is a cytokine that binds to its receptor and can result in the activation of caspase-8 under the appropriate circumstances in

some cell types. We investigated this apoptotic pathway because it has been documented that the production of TNF-α by skeletal muscle increases with age[60]. TNF-α secreted by skeletal muscle acts as an autocrine hormone, binding to its receptor on the cell that it was secreted from. Whether or not binding of TNF-α to its receptor results in caspase activation or what conditions are required for TNF-α to be an apoptotic signal in skeletal muscle are unknown. Despite reports of elevated TNF-α, caspase-8 expression nor its activated form changed with age. Furthermore, we did not detect any differences in the caspase-8 inhibitor, cFLIP. These data may suggest that the TNF-α receptor-mediated pathway of apoptosis does not play a significant role in fiber loss with age.

Alterations of apoptotic pathways with caloric restriction. Caloric restriction had a general effect of reversing most of the age-related adaptations to apoptotic stimuli. Caloric restriction reduced the apoptotic index in aged animals and attenuated the activation of caspase-3. Although caloric restriction did not affect cytochrome c release, cytosolic ARC increased ~75% compared with the senescent rats. Moreover, the mitochondrial content of ARC decreased with caloric restriction. These data suggest that caloric restriction reduces ROS production and mitochondrial stress and prevents the translocation of cytosolic ARC to the mitochondrial membrane. These data are in concordance with a suppressed H_2O_2 production with caloric restriction.

Caloric restriction reduced the total AIF content occurring with age but did not significantly affect nuclear content. The decline in total AIF may be in response to the reduced demand for this oxido-reductase.

Caloric restriction also altered the endoplasmic-reticulum-mediated pathway. Caloric restriction reduced procaspase-12 and its cleaved product, strongly suggesting that caloric restriction reduces stress in the endoplasmic reticulum. These results are novel in that no data thus far have described the effects of caloric restriction on ER stress or calcium homeostasis. A mechanism of caspase-12 activation during ER stress is cleavage of the procaspase by caspase-7. We show that the activated form of caspase-7 is also reduced with caloric restriction. These results imply that the apoptotic pathway induced by ER stress with age is suppressed with lifelong caloric restriction.

Finally, caloric restriction did not affect the TNF-α receptor-mediated pathway. Caloric restriction did not alter the content of procaspase-8 or the cleaved product. Moreover, caloric restiction did not affect the content of cFLIP, the associated inhibitor of caspase-8.

Consistent with a slowing of the aging process, we have shown that caloric restriction reduces apoptotic stimuli in aged muscle, such as mitochondrial H_2O_2 production and ER stress. Furthermore, the rate of apoptosis is suppressed as well as several apoptotic pathways that may be responsible for the phenomenon. Attenuation of apoptosis by caloric restriction may be preserving skeletal muscle fibers that are lost with the normal aging process.

Final Conclusions

These are the first studies conducted to investigate the role of apoptosis in sarcopenia. To give additional insight into the effects of aging on apoptosis, we used caloric restriction as a proven intervention to slow the aging process. We expected

that if apoptosis is involved with aging, caloric restriction would reduce apoptotic stimuli, the rate of apoptosis, and reverse age-related alterations in apoptotic signaling pathways. Caloric restriction did indeed meet our expectations. The data support that apoptosis is involved with the loss of muscle cells with age. The first apoptotic signaling pathway studied was the mitochondrial-mediated pathway. Although mitochondrial dysfunction increases in aging skeletal muscle, it appears that the pathways involved with age-related apoptosis are not dependent upon mitochondrial cytochrome c release and the associated caspase cascade via caspase-9 and caspase-3, nor the release of AIF as a caspase-independent mechanism. Skeletal muscle mitochondria adapt with age to prevent release of apoptogenic proteins by altering ARC localization, antioxidant defenses such as glutathione, and/or Bcl-2 family proteins. The activation of the mitochondrial-mediated caspase cascade is limited by the activation of procaspase-9 in skeletal muscle, which may be due to the presence of endogenous caspase inhibitors, such as XIAP. Age-related increases in the content of XIAP may be another protective mechanism against mitochondrial-mediated apoptosis. The second pathway studied was the endoplasmic-reticulum-mediated pathway. Aged skeletal muscle is associated with severe ER stress and calcium dyshomeostasis as evidenced by a dramatic increase in procaspase-12 content. The third pathway studied was the receptor-mediated pathway. It appears that the receptor-mediated pathway via TNF-α is not affected by age or caloric restriction and therefore not significantly contributing to age-related cell loss. Of the apoptotic pathways studied, that involving ER stress and caspase-12 may play a prominent role in the loss of skeletal muscle fibers with age. Further research should focus on the

endoplasmic-reticulum-mediated pathway elucidating the scope of caspase-12 involvement in age-related apoptosis and will clarify the sequence of events leading to cell death and sarcopenia.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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